

Monographs on Biochemistry

NUCLEIC ACIDS
THEIR CHEMICAL PROPERTIES AND
PHYSIOLOGICAL CONDUCT

BY
WALTER JONES, PH.D.

MONOGRAPHS ON BIOCHEMISTRY

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GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason, an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield full and independent information of the work which has been done upon the subject.

It has been decided as a general scheme that the volumes first issued shall deal with the pure chemistry of physiological products and with certain general aspects of the subject. Subsequent monographs will be devoted to such questions as the chemistry of special tissues and particular aspects of metabolism. So the series, if continued, will proceed from physiological chemistry to what may be now more properly termed chemical physiology. This will depend upon the success which the first series achieves, and upon the divisions of the subject which may be of interest at the time.

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NUCLEIC ACIDS

THEIR CHEMICAL PROPERTIES AND PHYSIOLOGICAL CONDUCT

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PREFACE TO THE SECOND EDITION

A COMPARISON of the different editions of a text-book often brings out interesting historical matters.

When the first edition of this monograph appeared, the nucleotide structure of plant nucleic acid, though firmly grounded on the four nucleosides, was nevertheless in the hypothetical stage; and this structure was the point of dependence for the analogous nucleotide structure of animal nucleic acid, so that the two structures must survive or fall together. The four hypothetical nucleotides have now been prepared from plant nucleic acid, and analogies dealing with the fundamental chemical and physiological properties of the two nucleic acids have been so frequently found that at the present time interest in the two structures is directed only to minor points.

A most important development has also occurred on the physiological side. After it was shown that the purine fermentation is markedly different for different animal species and that the purine ferments have a successive embryonic development, efforts were of course directed to the discovery of evolutionary relations. After thorough examination it became certain that the organism of the monkey contrasted sharply with that of man and resembled closely that of the lower animals. This was a little unexpected, as the organs of the rabbit and guinea-pig had been found to coincide throughout. However, the conclusion was accepted without hesitation: in fact every one was rather pleased, and even took the trouble to emphasize this notable difference between man and the monkey. But a subsequent examination of apes put the evolutionary matter in a somewhat different light.

In regard to the most striking factor of purine metabolism (ability to convert uric acid into the more soluble allantoin) the lower animals closely resemble one another. Their "uricolytic index" is high. But the uricolytic index of man and the

ape is zero: they cannot convert uric acid into allantoin. Therefore the gap occurs between the ape and the monkey, not between the ape and man, which constitute a class by themselves.

W. J.

PREFACE

THE nucleic acids constitute what is possibly the best understood field of Physiological Chemistry, yet so far as is known to the writer no treatise has yet appeared which deals exclusively with this subject. Our information must be acquired either from widely scattered and often conflicting original articles which reveal order only by the application of critical ability, or from incidental chapters of general texts which appear to have been added more for completeness than for the information which they contain. Under these conditions the appearance of a special volume is rather to be expected than explained.

In the present monograph an attempt has been made to give a comprehensive view of the field as it exists to-day and at the same time to preserve historical order so far as it concerns individual priority. At some points this has been found difficult and at others was made possible only by a trifle of repetition. It may appear that the method of treatment is based upon certain rather radical opinions. This is to some degree true. However, these opinions are not personal, but can be found stated or clearly implied in all of the more recent contributions. Their acceptance makes possible a clear exposition of the subject and their denial affects only the arrangement, not the materials, of this monograph.

W. J.

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PART I.

THE CHEMICAL PROPERTIES OF NUCLEIC ACIDS.

CHAPTER I.

INTRODUCTION.

Nucleoprotein, Nuclein, Nucleic Acid.

THE early development of nearly every scientific subject is marked by a set of conditions under which it is extremely difficult or even impossible to distinguish the important from the unessential, and unfortunately any misapprehensions which in consequence arise are likely to be so engrafted upon the nomenclature as to perpetuate themselves automatically.

The discovery of nucleic acid happened at a time when the chemistry of the proteins was little understood and when everything incapable of being otherwise rationally explained was ascribed to the proteins as the somewhat mysterious agents of all physiological complexity. The protein molecule was commonly represented with a sugar group, the brown pigments of the body were referred to the proteins of the blood, uric acid was regarded as an intermediate product of protein metabolism, and it was to be expected that nucleic acid with its high nitrogen content would be connected with this common source of nitrogen compounds. But the proteins and nucleic acid might easily have been disentangled had the subject not become confused by the supposed constitutional ability of the so-called "nucleoproteins" to produce intravascular clotting. This unfortunate causal association of two things which have nothing whatever to do with one another gave rise to a growth of literature that was highly respected in its day and even at present serves to impress many scientists. "Nuclein," a rather definite term from the pen of Miescher, became expanded to "nucleoprotein" by subsequent writers. The nucleoproteins were then subdivided for rather trivial reasons, and the components of this subdivision were confused with one another in a way that suggested chaos when Kossel rescued "nucleic acid" as the essential factor or "prosthetic group" of all nucleins and nucleoproteins.

Even at present the terms nuclein and nucleoprotein suggest something a little disquieting, but all difficulty disappears when the original sources of these terms are examined in the light of modern discovery.

In the year 1868 Friedrich Miescher [1868] undertook a chemical examination of pus cells. Surgical bandages, secured from a neighbouring clinic, were extracted with a dilute solution of sodium sulphate, and the heavy pus cells thus obtained were easily separated from adherent serum and salt solution by careful decantation. The cells, still intact, were then submitted to the digestive action of artificial gastric juice which dissolved the protoplasm, leaving the more resistant nucleus as an insoluble grey powder, so that cell nuclei free from protoplasm became available for chemical study. Upon treatment of these insoluble nuclei with dilute sodium carbonate a solution was obtained in which acetic acid produced a flocculent precipitate which was found to contain phosphorus and responded to protein colour tests. This substance, to which Miescher gave the name nuclein on account of its origin, was the first known member of what is now a comparatively large class of substances obtainable from the nuclei of animal and plant cells. Hoppe-Seyler [1871] prepared one from the nuclei of yeast cells, and Kossel [1881] afterwards prepared another from the red-blood corpuscles of birds. All of these nucleins are insoluble acids which form soluble sodium salts. They respond to the protein colour reactions, but differ from proteins in the phosphorus which they contain and in *the resistance which they offer to the solvent action of artificial gastric juice.*

Shortly after the completion of his work with pus cells, Miescher removed from the laboratory of Hoppe-Seyler in Tübingen to assume control of the department at Basel where he became intensely interested in the life of the Rhine salmon in fresh water (see Miescher [1897]). He was able to prove the older suspicion that these animals never partake of food in their ascent of the Rhine from the sea to the spawning beds; for with rare and easily explained exceptions the alimentary canal was found free from food detritus, and the digestive fluids were as a rule inactive. As the muscle tissue had greatly decreased during the Rhine journey, while the organs of reproduction had grown enormously, it is necessary to conclude that eggs and spermatozoa had been formed from muscle protein.

During the spawning season the spermatic fluid or *lachsmilch* can be obtained from these fish in great quantity, and when expressed from the *vas deferens* of a living fish, consists of little more than spermatozoa suspended in a dilute salt solution. The spermatozoa are composed of head, tail and middle part, it being especially

characteristic that the two latter parts together make up a mass that is insignificant in comparison with the mass of the head, while the tails are threads of extreme fineness which dissolve easily in acetic acid, leaving the heads as insoluble granular masses.

From comparative histological studies of the growing testicle and also from other considerations we are led rather directly to the conclusion that the spermatozoa head is to be regarded as a metamorphosed nucleus, so that Miescher was in possession of material in great quantity admirably adapted to a chemical examination of the cell nucleus. He found the spermatozoa heads free from protein, and *made up almost exclusively of a single chemical individual—a salt of an organic base rich in nitrogen and of an organic acid containing phosphorus* (Miescher [1874, 1]). The organic base was protamine; the acid, nucleic acid. While Miescher had previously isolated “nuclein” from pus cells, its demonstration lacked the clearness which characterizes his discovery of protein-free nucleic acid in the spermatozoa heads, and does not admit of the corollary that nucleic acid is formed in the body from the decomposition products of protein.

Miescher's work has been experimentally tested many times, and has been submitted to the closest critical examination, only to be found without a flaw (Schmiedeberg [1896]: [1900]). He was fortunate enough or wise enough to take advantage of the rare opportunity for scientific investigation which was afforded by the Rhine salmon, and became at a stroke the discoverer of both protamine and nucleic acid.

Miescher came close to another discovery of no less importance than the isolation of nucleic acid from cell nuclei. Upon warming a specimen of protamine with nitric acid a yellow spot was formed which changed to bright red when moistened with alkali. Appreciating the significance of the reaction, Miescher [1874, 2] asked Piccard to make an examination of salmon sperm for purine bases. Piccard [1874] made successive extractions of salmon spermatozoa with hydrochloric acid of increasing strength. The first extract contained only protamine; but the final extract, made with boiling acid, produced the well-known gelatinous purine precipitate with silver nitrate in ammonia and was found to contain considerable quantities of *guanine* and *hypoxanthine*. Considering the analytical methods at his disposal, Piccard's results are admirable; guanine was correct, and adenine, at that time unknown, was always mistaken for hypoxanthine. But unfortunately Piccard added that the composition of salmon sperm as given by Miescher must be revised to include guanine and hypoxanthine, which are to be ascribed partly to nuclein and *partly to protein*. He

had evidently forgotten that Miescher made his colour reaction with a substance that was free from protein, and the discovery of the "alloxuric bases" was therefore left for another.

Kossel observed that purine derivatives are always formed from nucleins when these substances are submitted to the action of hydrolytic agents, and he understood clearly that the bases originated from the "prosthetic group" (nucleic acid) and not from the protein of the nuclein. He first found hypoxanthine [1879] and a trace of xanthine [1880], then guanine [1882]: [1883-84], and finally adenine [1886]: [1888, 1]. It should be stated that nucleic acids by hydrolysis produce only the two *amino*-purines, guanine and adenine, and that the *ary*-purines formerly observed were always secondary products from these. As Kossel had not yet discovered adenine it is but natural that he should have mistaken the substance for hypoxanthine, while the trace of xanthine which he sometimes found was probably a laboratory product formed from guanine in the execution of the Neubauer method [1867] which was employed. Kossel's priority, however, is not in the least in question, for he himself soon found suitable methods of separating and identifying the bases (Schindler [1889], Bruhns [1890]).

Kossel's discovery of the "alloxuric bases" was of the utmost importance.

1. It gave character to nucleic acid and furnished a definition of the substance by which it could be distinguished from proteins and other constituents of the body.

2. It made possible a study of the distribution of nucleic acid in the body without actually separating the cell nucleus from the protoplasm (Kossel [1881]: [1882-83]).

3. It furnished a method of distinguishing true nucleins from pseudo-nucleins. A substance of the latter class had been prepared from milk by Lubavin [1870] and another from egg-yolk by Bunge [1885]. These pseudo-nucleins resemble the true nucleins in that they contain phosphorus and are resistant to the action of pepsin, but they are not constituents of the cell nucleus nor do they yield purine bases (Kossel [1891]).

4. It furnished the refutation (Kossel [1893]) of the persistent claims of Liebermann [1888, 1]: [1888, 2]: [1890], Pohl [1889], and Malfatti [1892], that nucleins are merely compounds of protein with metaphosphoric acid.

5. It suggested in no uncertain way a chemical connexion between the cell nucleus and urinary uric acid, and was thus the foundation of many fruitful investigations in experimental medicine.

But the relation of nuclein to protein remains to be considered. The presence of a salt, protamine nucleate, in the metamorphosed nucleus suggests the presence of protein nucleate in the original nucleus; or that nuclein is simply a salt of protein and nucleic acid.¹ This assumption cannot be definitely proven, but is supported by a large amount of evidence, and any other conclusion leads to complication without purpose.

1. Nucleic acids are polybasic acids and proteins are polyacid bases, so that a large number of salts of the two substances are to be expected. One of these salts will have a greater resistance to pepsin than the others, and will be formed as an end product of the action of pepsin upon any other salt having a greater proportion of protein in its composition.

2. The nucleins are not entirely unaltered by pepsin, they are only somewhat resistant to its action (Umber [1901]).

3. Artificial nucleins resistant to the action of pepsin are formed as immediate precipitates when faintly acid solutions of protein and nucleic acid are brought together (Milroy [1896]).

4. Brief contact of nucleins with dilute caustic soda renders the protein part precipitable by acetic acid (Altmann [1889]).

5. From solutions of nuclein, picric acid precipitates the protein, leaving the nucleic acid in solution (Levene [1901]).

6. The presence of protein is apparently without influence upon the decomposition of nucleic acid by the action of ferments.

7. A solution containing both nucleic acid and protein has an optical rotation equal to the algebraic sum of the rotations of the two constituents (Osborne [1903]).

In the year 1889 Altmann [1889] described a method of preparing protein-free nucleic acids from animal tissues and from yeast, when the interest which had previously been taken in nuclein began to decrease. Subsequently, Kossel and Neumann [1894, 1] devised a method of preparing nucleic acid from the thymus gland. Under the most favourable conditions their procedure gives only passable results and requires the greatest care in its execution, but it furnished Kossel and Neumann the material for their wonderful researches on nucleic acid and made Kossel considerably less interested in nuclein. Finally Neumann [1899] showed how nucleic acid can easily be prepared from all of its common sources except yeast (Kowalevsky [1910]) and in such quantity as to place the substance within easy reach of

¹ Like the heads of fish spermatozoa, the tubercle bacillus contains protamine nucleate (Ruppel [1898]).

every one. From this time it is difficult to find a serious reference to "nuclein" in the literature. Wherever the term is used it is employed as the adjective for nucleic acid. (The "nuclein metabolism" means the metabolism of nucleic acid.)

α -Nucleoproteins.

Questions relative to nucleoprotein become greatly simplified if one distinguishes sharply between two classes of these substances which are called *α -* and *β -nucleoproteins*. The *α -nucleoproteins* are prepared from cloudy aqueous gland extracts by precipitation with acetic acid. The substances thus precipitated can be gotten into an emulsion with very dilute caustic soda, and by alternate precipitation with acetic acid and solution in alkali the product can in a measure be "purified". In this way Wooldridge [1881]: [1886] prepared substances which produced intravascular clotting when injected into animals;¹ but Halliburton and Brodie [1894-95] observed that this physiological property was lost when the substances were repeatedly submitted to the process of purification. On the other hand, Martin [1893] has shown that snake venom can produce intra-vascular clotting although it is in no way chemically related to nucleoprotein. The method of preparing nucleoproteins is so crude that one is not inclined to attribute their individual properties to the substances themselves but rather to the impurities which they contain.² Various *α -nucleoproteins* have been prepared by Halliburton [1892]: [1893]: [1895], Gourlay [1894], Halliburton and Brodie [1894-95], Forrest [1894], Umber [1900], Gamgee and Jones [1903], Jones and Whipple [1902], and others.

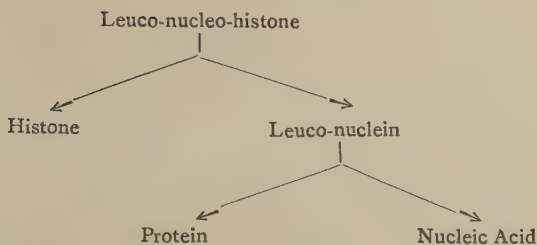
While all of these *α -nucleoproteins* are of importance only in so far as they contain nucleic acid, a special interest has attached to the closely studied nucleoprotein of the thymus. While engaged in a study of the part played by leucocytes in blood coagulation, Lilienfeld [1892, 1] found that the leucocytes of the thymus contain a peculiar nucleoprotein which he called leuco-nucleo-histone. By treatment with acetic acid the substance lost histone and leuco-nuclein was formed, which in turn could be split into protein and nucleic acid. These changes were represented thus:—

¹ Due simply to the thrombase entrapped in them.

² Nucleic acids form precipitates with a great variety of substances; diphtheria toxin, ricin, etc. (Tichomiroff [1895-96]).

INTRODUCTION

7



In a subsequent communication Lilienfeld [1892, 2] reconsidered his experimental findings and changed his nomenclature, but the implication remained that in thymus-nucleoprotein the nucleic acid is in combination with two different proteins. If such a finding could be sustained a particular meaning would be given to the expression "nucleoprotein"; but from the unanimous testimony on this point of Malengreau [1900], Huiskamp [1901]: [1903], and Bang [1904] we are to conclude that Lilienfeld was dealing with a mixture of two or more substances, and we are left with no more information than that proteins and histones alike form combinations with nucleic acid which differ from one another in the relative ease with which they can be precipitated by various reagents.

Nucleo-histone came into physiological chemistry on a high wave of enthusiasm which has served for its preservation for many years; but the recent work of Steudel [1913], [1914] would seem to make a final disposal of the substance. He finds that specimens of nucleo-histone have a composition that depends entirely upon the method used in their preparation.

After a careful examination of a mass of badly tangled evidence upon the subject one is likely to conclude that α -nucleoprotein consists of various salts of protein with nucleic acid in which the protein is in excess; that when the substances are submitted to the action of pepsin-hydrochloric acid part of the protein is digested away leaving a mixture of more acid salts indefinitely called nuclein, and more resistant to the action of the ferment. The terms nucleoprotein, nuclein and nucleic acid therefore express a relation which means little more than that conveyed by the terms basic lead acetate, lead acetate and acetic acid. At least this is the greatest concession that can be made, for in reality "nucleoprotein" means rather "a method of preparation" than a chemical substance. A specimen was actually looked upon as identical with trypsin (Hammarsten [1894]): all are probably contaminated with glucothionic acid (Levene and Mandel [1906, 1]).

β -Nucleoproteins.

The first member of this group was prepared by Hammarsten from the pancreas [1894]. Finely ground glands were suspended in water and the mixture quickly heated to boiling. The pale yellow transparent fluid was filtered from the bulky dark brown coagulum and treated with acetic acid, when the β -nucleoprotein was thrown out as a white flocculent precipitate. The difference between this procedure and that employed in the preparation of α -nucleoprotein is notable. Most of the glandular constituents are thrown down with the heat coagulum and the rest are left in solution, when the nucleoprotein is precipitated with acetic acid.

Upon hydrolysis of β -nucleoprotein, Hammarsten obtained a reducing pentose and but one of the alloxuric bases, viz. guanine: the substance is thus sharply distinguished from α -nucleoprotein.

Similar β -nucleoproteins have been obtained from the mammary gland (Odenius [1900]), the spleen (Jones and Rowntree [1908]), and the liver (Levene and Mandel [1908]), and the wide occurrence of pentose in animal tissues (Blumenthal [1897], Bang [1897], Wohlgemuth [1904]) is almost certainly to be ascribed to the β -nucleoprotein which they contain. But it should not be understood that β -nucleoproteins are protein salts of *nucleic acid*, nor that they are constituents of cell nuclei. When the protein is removed from them there remains a substance called guanylic acid which yields phosphoric acid and guanine, but is not a nucleic acid in the narrow sense of the term as will be explained in Chapter III.

To regard α -nucleoproteins and nucleins as protein salts of nucleic acid is not to detract from the important writings of those who formerly looked upon the matter in a different light (Pekelharing [1895]). Kossel kept alive a keen interest in nucleins so long as he was compelled to work with them; but he was quick enough to abandon nuclein with its protein complexity for the "prosthetic group," which could be made to yield definite results by the application of the methods of organic chemistry.

CHAPTER II.

THE FUNDAMENTAL GROUPS OF NUCLEIC ACIDS.

Animal and Plant Nucleic Acids.

OUR chemical and physiological knowledge of nucleic acids has been acquired to a very considerable extent from researches executed with two substances, one of which is prepared from yeast and the other from the thymus gland. These two nucleic acids are the best-known members of two sharply defined groups into which all substances of this class may be divided, and indeed there is a well-formed and constantly growing opinion among physiological chemists that all nucleic acids are identical with one or the other of these two compounds. This opinion is based upon the most firmly established facts in our possession and furnishes a rational explanation of a mass of otherwise discordant material, but a superficial reading of the older contributions might lead one to an entirely different conclusion. This is because a number of disturbing factors have continually operated to confuse experimental findings. Investigations have been conducted with impure material at a time when dependable methods of separating decomposition products were not available. Products of secondary decomposition were assumed to be primary, and substances formed from adherent impurities have been ascribed to the nucleic acids themselves. But if such literature, with its unavoidable errors, be reviewed in the light of our most recent knowledge, all confusion disappears, and we come to understand quite clearly that there are but two nucleic acids in nature, one obtainable from the nuclei of animal cells, and the other from the nuclei of plant cells.¹ In examining the evidence one naturally takes into consideration that experimental exactness in such a field is entirely out of question and that concurrent tendencies are all that can be reasonably demanded.

A number of investigators have taken great pains to perfect methods of preparing nucleic acids sufficiently pure for elementary

¹A possible exception is to be found in the nucleic acid of fish-eggs, which is of animal origin and yet resembles plant nucleic acid so far as its examination has extended (Levene and Mandel [1906, 2], and Mandel and Levene [1905-6]).

chemical analysis. Schmiedeberg [1900] isolated the substances as copper compounds by a method that eliminates adherent carbohydrates. Analysis of fifteen of these copper preparations by Herlant [1900], Alsberg [1904], and Schmiedeberg [1907] showed a striking resemblance in elementary composition between various animal nucleic acids which is very different from the composition of yeast nucleic acid (Boos [1906]); and Matthews [1897] found the composition of the nucleic acid of invertebrates close to that of salmon nucleic acid but different from that of yeast nucleic acid.

Levene and Mandel¹ prepared animal nucleic acids from a number of sources. They employed copper compounds to some extent in their method of purification, but found that nucleic acids could be most effectively separated from proteins by precipitation of the latter with picric acid (Levene [1900]). The great differences both in empirical composition and decomposition products shown by their preparations have probably been the chief reason for assuming individual differences between animal nucleic acids. But it should be noted that Levene and Jacobs in their most recent writings draw no distinction whatever between the animal nucleic acids of various origin. In one publication [1912, 1] they do not even consider it necessary to name the origin of the animal nucleic acid which they employ, and in a second publication [1912, 2] they announce a provisional structure of *thymus* nucleic acid from data acquired with the nucleic acid of *fish sperm*.

Until the year 1902 all our knowledge of plant nucleic acid had been gotten from yeast nucleic acid, and in that year the contribution of Osborne and Harris on tritico-nucleic acid appeared [1902]. They obtained the substance from the wheat embryo by a variety of methods, and having in hand about 750 grams of material they were in a position to make an exhaustive examination of plant nucleic acid. Their elementary analysis agreed closely with the analysis of yeast nucleic acid and differed markedly from that of animal nucleic acid; in fact after a very thorough examination of their various preparations, they were inclined to believe the two plant nucleic acids identical.

Ultimate chemical analysis is of course not a dependable method of showing the identity of two complex chemical compounds, but all the evidence points in one direction, and is supported by a study of the physical properties of the substances.

¹ Levene [1900]: [1901]: [1902-3, 1]: [1903, 1]: [1903, 2]: [1903, 3]: [1903, 4]: [1904-5]: [1905]. Levene and Mandel [1906, 2]: [1906-7]: [1908, 1]. Mandel and Levene [1905]: [1906].

From an optical examination of the nucleic acids of thymus, pancreas, and spleen, the following observations have been made (Jones [1908]):—

1. Solutions of the sodium salts of all three nucleic acids have the same optical rotation in any given concentration.
2. The optical rotation varies with the concentration and according to the same law in the case of each nucleic acid.
3. The variation in rotation by dilution with ammonia is different from that by dilution with water, but is the same with all three nucleic acids.
4. The variation in rotation by dilution with acid obeys still a third law, and here again the three nucleic acids behave alike.
5. The variation in rotation by dilution with any solvent is parallel to a variation in the viscosity of the solution. In this respect also the three nucleic acids are scarcely distinguishable from one another.

The actual identity of two complex chemical substances can never be proved, but one may come to a point where it is unprofitable to make further comparisons. It would seem that this condition has been reached with animal nucleic acids when a prominent contributor suggests that the designation of animal nucleic acids by the names of the glands from which they are obtained is as superfluous as would be the application of a similar nomenclature to lecithin (Steudel [1908]). It is therefore necessary to discuss only two nucleic acids in order to have an understanding of them all.

The Final Hydrolytic Products of Nucleic Acids.

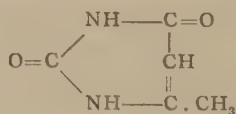
Thymus nucleic acid was first chemically examined by Kossel and Neumann [1893]: [1894, 1]. Kossel had previously found that nucleic acids of different origin are markedly different from one another in respect to the relative quantities of the purine bases which they yield on hydrolysis [1894]: [1891]. Yeast nucleic acid had been found (as Kossel supposed) to yield four of these compounds, guanine, adenine, xanthine and hypoxanthine, but Kossel and Inuko [1894] had obtained only three purine bases from the nucleic acid of steer sperm. Now most unexpectedly Kossel and Neumann find only adenine among the hydrolytic products of thymus nucleic acid. Thinking it improbable that so large a number of nucleic acids actually exist in animal and plant tissues as the supposed facts indicated, Kossel and Neumann assumed that there are in fact but four nucleic acids each of which yields a single purine base, and

that the various preparations of nucleic acid examined are in reality variable mixtures of these four. Having found in the thymus gland what appeared to be "adenylic acid" they suggested the possible discovery of "guanylic acid". But Kossel's work which at this point was slightly confused was not likely to remain obscured, and he soon found that thymus nucleic acid yields both amino-purines, guanine and adenine. The two purine bases are formed from thymus nucleic acid by the application of mild hydrolytic agents, and a more resistant ill-defined substance remains called thymic acid (see also Steudel and Brigl [1911]: Feulgen [1918]: Feulgen and Landmann [1918]). By more violent methods of hydrolysis, as with 40 per cent. sulphuric acid at 150° under pressure, Kossel and Neumann found that the substance undergoes complete hydrolysis. The purine bases are destroyed and the following substances are additionally produced:—

- | | |
|--------------------|---------------------|
| 1. Thymine. | 4. Formic Acid. |
| 2. Cytosine. | 5. Ammonia. |
| 3. Lævulinic Acid. | 6. Phosphoric Acid. |

The lævulinic acid was thoroughly identified and was looked upon as a secondary product formed from a hexose group in the nucleic acid molecule.

Thymine was easily isolated. It is a beautifully crystalline compound (Gulewitsch [1899]) which can be purified by repeated crystallization from hot water. Kossel and Neumann assigned to it its correct formula, $C_5H_6N_2O_2$, and observed that the substance has the *empirical composition* of 4-methyl-uracil (Behrend [1885]):—



Cytosine was not so easy to isolate. However it was found to be a base which forms well-crystallized salts, including a crystalline difficultly soluble picrate. The substance was accurately defined by Kossel and Neumann, but they were unable to secure material for satisfactory analyses and provisionally assigned the formula $C_{21}H_{30}N_{16}O_4$. The correct formula, $C_4H_5N_3O$, was assigned later by Kossel and Steudel [1902-3].

Yeast nucleic acid was first obtained by Altmann [1889]. He made no chemical examination but simply showed that from yeast, as from various animal glands, a compound of the nucleic acid type may be obtained by the application of his general method of preparation. The first chemical studies of yeast nucleic acid were made

by Kossel [1893] who found among its hydrolytic products the purine bases, or alloxuric bases, which he had previously obtained from animal nucleic acid. But specific differences between the two groups of nucleic acids were emphasized. From yeast nucleic acid was obtained a reducing carbohydrate which produced furfural, formed an osazone melting at 150° and was not fermentable with yeast. Kossel had no hesitation in pronouncing the substance a pentose, and called special attention to the fact that pentose is never formed similarly from animal nucleic acid.

In the presence of the pentosazone was found a hexosazone melting at 205° which led to the supposition that yeast nucleic acid (nuclein) contains both a pentose and a hexose group, but the hexose was afterwards traced to an impurity (Boos [1908-9]).

By partial hydrolysis of yeast nucleic acid with alkalis in the cold the purine bases were partly removed, leaving a substance rich in phosphorus which Kossel called plasmic acid and which was more accurately examined later by Ascoli [1899]: [1900-1, 1]. Continued action of alkali on plasmic acid finally removed all purine bases and the carbohydrate leaving a compound which Kossel called polymetaphosphoric acid [1893]. Somewhat later, uracil (Ascoli [1900-1, 2]) and cytosine (Kossel and Steudel [1903]) were obtained from yeast nucleic acid, by acid hydrolysis.

Thus Kossel discovered the hydrolytic products of nucleic acid. Various methods of preparing nucleic acids have since been adopted and refined procedures for the separation and identification of their hydrolytic products have been evolved, but no nucleic acid has ever been found which gives results different from those obtained with thymus nucleic acid and yeast nucleic acid by Kossel and his co-workers.

Hydrolytic Products of Nucleic Acid.

<i>Of Plant Origin.</i>	<i>Of Animal Origin.</i>
Phosphoric Acid	Phosphoric Acid
Guanine	Guanine
Adenine	Adenine
Cytosine	Cytosine
Uracil ¹	Thymine
Pentose	Lævulinic Acid

Nucleic acid is therefore a dehydrolysed product of phosphoric acid, carbohydrate and four nitrogenous ring compounds. These

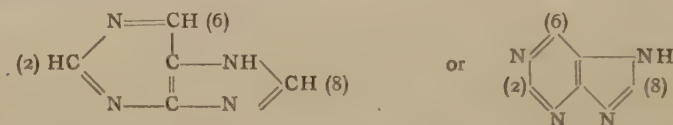
¹ The literature contains descriptions of animal nucleic acids which yield uracil, but it is now unanimously conceded that uracil in this connexion is secondary to cytosine:—



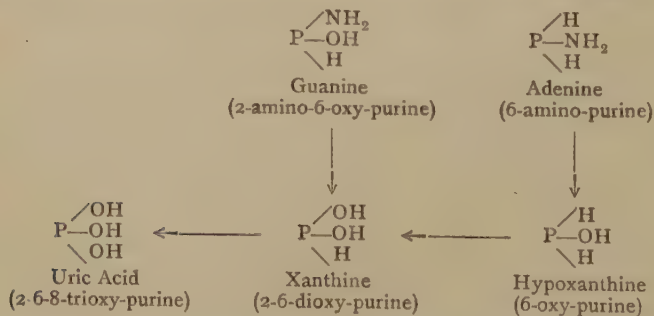
eight substances constitute the fundamental groups of nucleic acids. Kossel introduced four of them into chemistry (adenine, cytosine, thymine, uracil). It is necessary to make a close examination of them all.

The Purine Derivatives of Nucleic Acids.

In view of the communications of Inouye [1906], Inouye and Kotake [1905], Mochizuki and Kotake [1904-5], and of Steudel [1904]: [1904-5]: [1905], it must be insisted that guanine and adenine are the only purine bases ever obtained as primary hydrolytic products of nucleic acid. Nevertheless, physiology is concerned with three others that are formed from these by metabolic processes: so that five purine derivatives are to be considered, guanine, adenine, hypoxanthine, xanthine and uric acid. Four of these compounds have long been known (Strecker [1861], Wulff [1893]) and have furnished material for some of the most important investigations of organic chemistry (Fischer [1907]), but the fifth (adenine) was more recently discovered by Kossel [1886] among the hydrolytic products of pancreas nucleic acid. All five are chemical derivatives of one mother substance, purine (Fischer [1898]), whose constitution is expressed by the formula:—

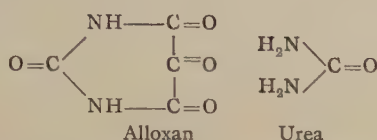


Representing the purine ring with its three replaceable hydrogen atoms by the abbreviated expression $\begin{array}{c} \diagup \text{H (2)} \\ \text{P} \text{—} \text{H (6)} \\ \diagdown \text{H (8)} \end{array}$, the relation of the five purine compounds to one another is seen in the following diagram:—

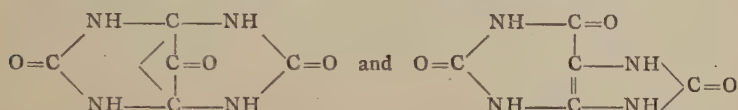


The exhaustive proof of these five structures would be entirely out of question here, and any such attempt is made superfluous by the

summary of Fischer [1907]. But for the proof of the essential purine structure two cardinal points will suffice. In 1838, Liebig and Wöhler [1838] obtained alloxan and urea by the oxidation of uric acid with nitric acid:—

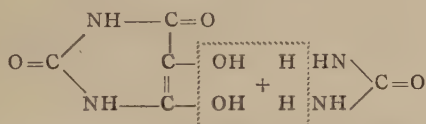


Medicus [1875] and Fittig [1877] both believed that this reaction limited the structure of uric acid to a choice between the two formulas:—



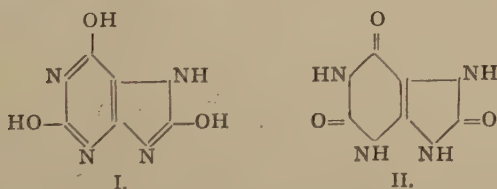
the only difference being in the points of union between the urea and alloxan rings. It seems that Medicus made a place for himself in history by his ability to guess the correct formula.

Behrend and Roosen [1889] put the matter beyond conjecture by their synthesis of uric acid from urea and 4-5-dioxy-uracil:—



a reaction which shows that uric acid has the unsymmetrical formula. The structure of uric acid is the key to the structure of other purine derivatives including purine itself.

The unsymmetrical double ring structure for uric acid is clearly proven, but there are two tautomeric formulas for the compound which meet this requirement and are equally well supported by the chemical conduct of the substance:—



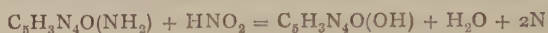
I. is the lactam formula whose principal support is the formation of *tri*-chlor-purine by the action of phosphorous pentachloride on uric acid. It is the more convenient of the two formulas when one is not dealing with methyl-purines.

II. is the lactim formula in which the replaceable hydrogen

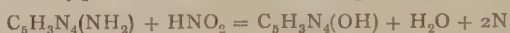
atoms are in combination with nitrogen and its principal support is the formation of methylamine by the oxidation of trimethyl-uric acid.

Since we are as much concerned with the chemical relation of the five purine compounds to one another as with the structure that underlies them all, the following points are of special importance :—

1. Fischer [1897, 1] has prepared the four bases from uric acid.
2. The amino-purines have been converted into the oxy-purines by the action of nitrous acid ; guanine into xanthine by Strecker [1858] :—

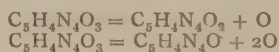


and adenine into hypoxanthine by Kossel [1886] :—

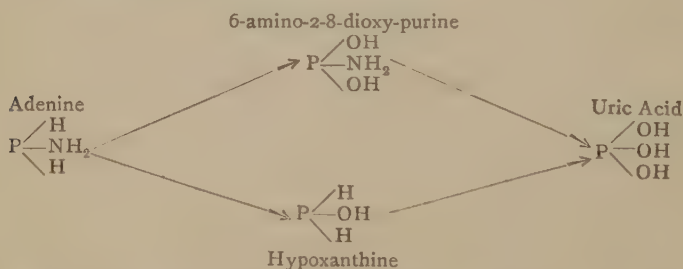


These transformations can also be produced by severe hydrolytic agents which accounts for the occasional finding of the oxy-purines among the hydrolytic products of nucleic acid.

3. Xanthine and hypoxanthine have both been prepared by direct reduction of uric acid (Sundwik [1912]) :—



The diagram given above shows how uric acid can be formed from either of the amino-purines by deaminization and subsequent oxidation, passing through the oxy-purines. But deaminization may follow, not precede oxidation, in which case the end products will be the same as before, but the intermediate products different. In the case of adenine the two possibilities are expressed as follows :—



In like manner guanine may theoretically pass to uric acid either through xanthine or through 2-amino-6,8-dioxy-purine. But the direct transformation of the amino-purines into one another is not theoretically possible ; the amino-group of adenine is in position six, while that of guanine is in position two, so that one need never be concerned about the formation of one of the compounds directly from the other.

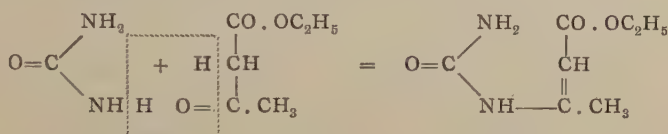
The Pyrimidine Derivatives of Nucleic Acids.

Physiological chemistry is especially concerned with three pyrimidine derivatives all of which became known to chemistry for the first time when Kossel and his co-workers found them among the hydrolytic products of nucleic acids:—

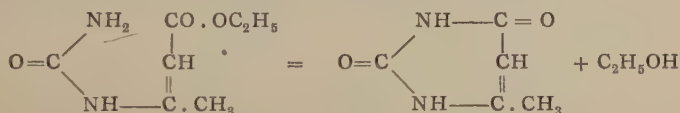
- (1) Cytosine.
- (2) Thymine.
- (3) Uracil.

Cytosine is produced by hydrolysis of both animal and plant nucleic acid, but thymine results only from animal nucleic acid and uracil only from plant nucleic acid (Ascoli [1900-1, 2]).

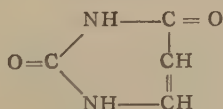
Pyrimidine derivatives were given special prominence by the work of Behrend [1885], who began his well-known synthesis of uric acid with a substance of this class. By condensation of aceto-acetic ether with urea, uramidocrotonic acid was formed:—



which by saponification produced 4-methyl-uracil:—



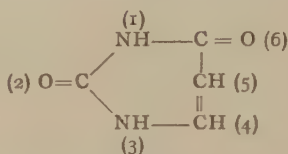
From this compound a large number of substances were prepared which Behrend looked upon as derivatives of the hypothetical substance, uracil:—



It was from the first apparent that the pyrimidine derivative which Ascoli [1900-1, 2] obtained from yeast nucleic acid has the empirical composition of the hypothetical uracil $\text{C}_4\text{H}_4\text{N}_2\text{O}_2$, and that thymine from thymus nucleic acid is either identical or isomeric with Behrend's 4-methyl-uracil. Moreover, the transformation of cytosine into uracil by the action of nitrous acid (Kossel and Steudel [1903]) proved that the two substances are corresponding oxy- and amino-compounds.¹

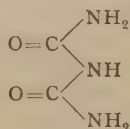
¹ The same conversion is brought about by violent hydrolysis which accounts for the supposed formation of uracil from animal nucleic acids (Kossel and Steudel [1902-3, 2],

The first structural investigations of the pyrimidine compounds were made with thymine. The contributions of Jones [1899] and of Steudel and Kossel [1900], while showing great similarity in the chemical properties of thymine and 4-methyl-uracil, completely excluded their possible identity. If a colour reaction together with the formation of urea can be accepted as proof, the later work of Steudel [1900]: [1901, 1] may be taken to mean that both thymine and uracil contain an alloxan ring, and the formula for thymine results by exclusion. For if the substance produces urea and not methyl urea its methyl group cannot occupy positions *one* or *three* :—

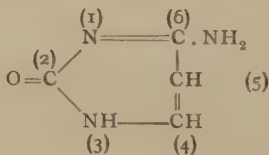


and if thymine is not identical with Behrend's 4-methyl-uracil it can only be 5-methyl-uracil.¹

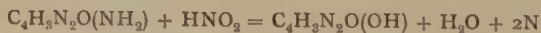
By oxidation of cytosine with barium permanganate, biuret is formed which in this connexion has only the significance of urea, but Kossel and Steudel [1903] take the reaction to mean that in cytosine the carbon and nitrogen atoms alternate :—



so that the amino-group cannot be situated in position five. As positions four and six are equivalent the structure of cytosine is determined :—

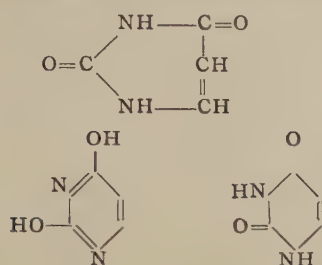


Steudel [1904]: [1904-5]: [1905], Levene [1903, 2]). The real existence of a uracil group in plant nucleic acids and its pseudo-existence in animal nucleic acids must have been very confusing.



¹ The substance which Schmiedeberg calls "nucleosine" [1896] is thymine (Kossel [1896-97]).

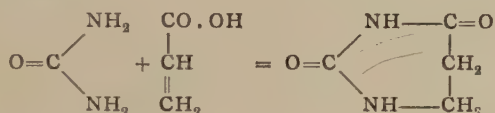
and from its relation to cytosine the formula for uracil follows¹ :—



It is at times difficult to say just what constitutes proof in structural chemistry. But whether upon sufficient evidence or not the formulas which Kossel assigned to these three pyrimidine derivatives have been confirmed by the conclusive synthetical work of Fischer and Roeder, Wheeler and Merriam, and Wheeler and Johnson.

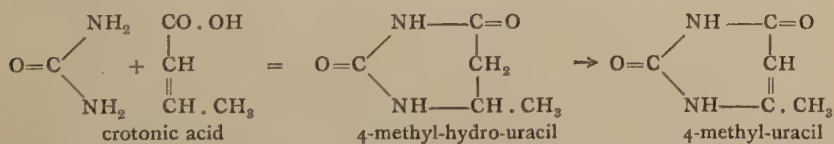
Syntheses of the Pyrimidine Derivatives.

By treatment of acrylic acid with urea Fischer and Roeder [1901] obtained hydro-uracil :—

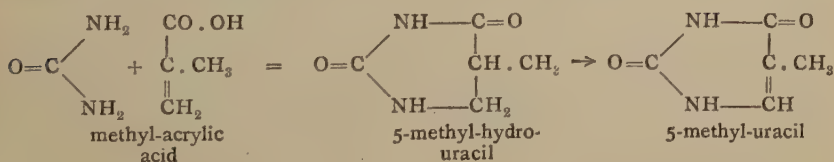


and by reduction of a bromine substitution product of the substance, uracil was formed, identical with the compound which Kossel and Ascoli had obtained by hydrolysis of yeast nucleic acid.

By a set of similar reactions crotonic acid gave Behrend's 4-methyl-uracil :—

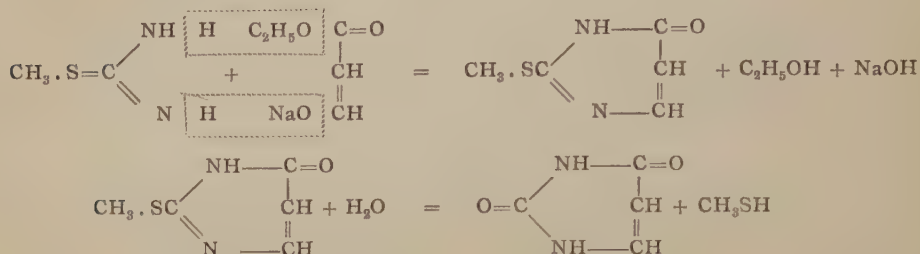


While from methyl-acrylic acid was obtained 5-methyl-uracil identical with thymine :—

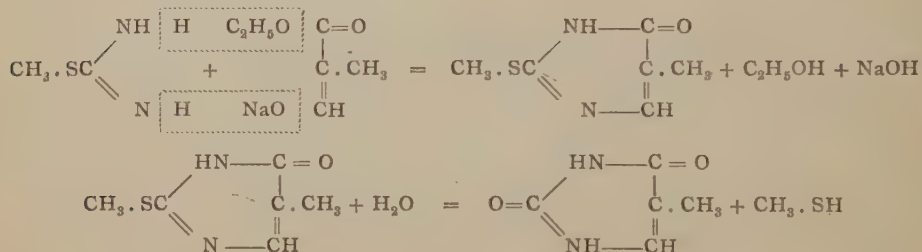


¹ Tautomerism exists among pyrimidine compounds as among purine compounds, and reasons for the use of two formulas for uracil are analogous to those considered in connexion with uric acid.

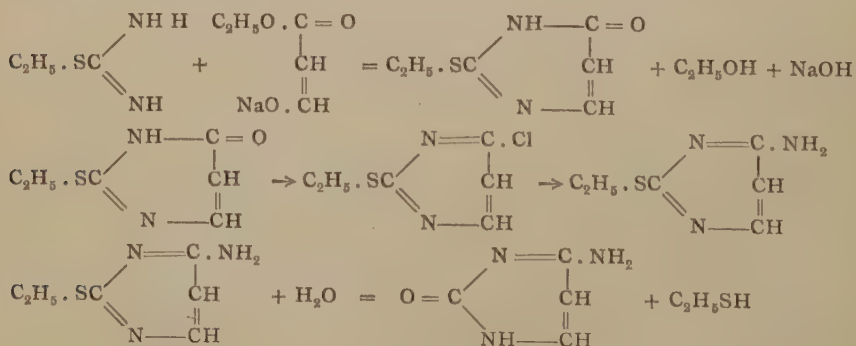
Wheeler and Merriam [1903] found that formyl-acetic-ether condenses with methyl-pseudothio-urea to form methyl-mercapto-uracil from which uracil is easily formed by saponification with boiling mineral acid:—



Starting with formyl-propionic ether they similarly obtained 5-methyl-uracil, identical with thymine:—



By the action of phosphorus pentachloride on 2-ethyl-mercapto-6-oxy-pyrimidine, Wheeler and Johnson [1903, 1] prepared 2-ethyl-mercapto-6-chlor-pyrimidine. Upon boiling this substance with alcoholic ammonia the corresponding amide was formed which in turn was converted by saponification into 2-oxy-6-amino-pyrimidine, identical with cytosine:—



The Primary Origin of the Pyrimidine Derivatives.

The possible secondary formation of the pyrimidine derivatives from the purine groups of nucleic acid was likely to be suggested on speculative grounds. Of thymine there can scarcely be a question for its methyl group precludes its formation from either guanine or adenine, but Burian [1907, 2] called both uracil and cytosine into question. In order to reproduce closely the conditions under which the pyrimidines are formed from nucleic acid, he heated mixtures of guanine, adenine, xanthine and hypoxanthine with sulphuric acid at 150° in the presence of carbohydrates. Without applying any method to the product which would remove the purine compounds he artlessly found a substance which he thought resembled cytosine both in properties and chemical composition, and a second compound which he pronounced identical with uracil. Osborne and Heyl [1908] showed that Burian's findings were without bearing upon the nucleic acid question even if they were true, and by repetition of Burian's experiments Steudel [1907, 2] found that the supposed pyrimidine derivatives were simply contaminated portions of the initial purine compounds.

Burian's contention belongs to that large category of hypotheses that depend for their acceptance more upon the difficulty or even impossibility of disproving them than upon any element of probability that they possess. However, the possible origin of the pyrimidines from the purines has been sufficiently disposed of by the preparation from yeast nucleic acid of four nucleosides and four nucleotides (see below).

The Carbohydrate Groups of Nucleic Acids.

By hydrolysis with boiling mineral acid, the plant nucleic acids produce a pentose whose discussion is best postponed until inosinic acid is taken up.

But the animal nucleic acids do not yield a hexose correspondingly, because the particular hexose is not stable under the conditions. By heating hexoses in general with sulphuric acid, lævulinic acid (β -acetyl propionic acid $\text{CH}_3\text{CO} \cdot \text{CH}_2\text{CH}_2\text{COOH}$) is formed and formic acid is usually produced at the same time



so that the formation of the two substances from animal nucleic acid indicates a hexose precursor, especially as lævulinic acid is not formed similarly from pentoses. Kossel and Neumann [1894, 1] found both lævulinic acid and formic acid among the products of severe hydrolysis

of thymus nucleic acid and drew the conclusion that the nucleic acid contains a hexose group. The crude products were extracted with ether and the viscous brown residue obtained by evaporation of the solvent was submitted to fractional distillation. At first formic acid passed over which was easily identified. The temperature then rose rapidly to the boiling-point of lævulinic acid (250°) when a colourless viscous distillate was obtained, which was easily soluble in water, alcohol and ether. An aqueous solution of the substance responded to the qualitative tests for lævulinic acid and formed with silver nitrate a crystalline precipitate whose analysis corresponded with the formula $C_5H_7O_3Ag$.

The substance has been obtained uniformly from animal nucleic acids (Noll [1898], Inouye [1894], Araki [1903]), but is never formed from plant nucleic acid.

It is of great interest that Steudel [1906-7]: [1907] has obtained a saccharic acid ($C_6H_{10}O_8$) by oxidising thymus nucleic acid with nitric acid.

More recently, Feulgen [1914, 3], [1918, 4] has claimed that the carbohydrate group of thymus nucleic acid is not a group of hexose ($C_6H_{12}O_6$) but of glucal ($C_6H_{10}O_4$) [Fischer, 1914]. His evidence, however, is not convincing. He states that thymic acid (an ill-defined partial hydrolytic product of thymus nucleic acid) darkens more rapidly when heated with mineral acid than one would expect of a hexose; and it happens that glucal darkens easily. Feulgen also finds that thymic acid gives a fuchsin colour-reaction which is given by glucal but not by hexose.

CHAPTER III.

PLANT NUCLEIC ACIDS.

Yeast Nucleic Acid.

FROM the time that Kossel made his early and conclusive experiments with yeast nucleic acid the substance received little attention until Levene [1909] described a method of purifying nucleic acids by precipitating them from concentrated aqueous solution with glacial acetic acid. Nucleic acid of yeast prepared in this way was found dextro-rotatory to polarized light and the rotation markedly influenced by the presence of alkalis as had been observed with thymus nucleic acid (Jones [1908]).¹ From elementary analysis of the purified product Levene assigned to yeast nucleic acid the formula $C_{38}H_{55}N_{15}P_4O_{32}$, and from a quantitative estimation of its hydrolytic products he decided that the substance contains equimolecular quantities of the four nitrogenous groups, guanine, adenine, cytosine and uracil. A complex formula was then assigned which from the defective and insufficient data upon which it was based could scarcely be taken seriously, but it has been greatly strengthened by the work of Levene and Jacobs. Before entering upon its discussion it is necessary to consider two substances, inosinic acid and guanylic acid, whose chemical conduct led to the structure of yeast nucleic acid.²

Inosinic Acid.

Inosinic acid was first obtained from meat extract by Liebig [1847]. From the amorphous acid he prepared a crystalline barium salt which

¹ For the variation in rotation of yeast nucleic acid with temperature, see Amberg and Jones [1911, 1].

² There is no doubt that the principal nucleic acid of yeast is the substance which has been described, but two very interesting matters remotely suggest the possible presence of at least traces of other nucleic acids. Mandel and Dunham [1912] obtained from yeast a crystalline compound of the formula $C_{11}H_{15}N_5O_5$ which produces adenine and lævulinic acid by hydrolysis. The substance is evidently adenine hexoside $C_6H_{11}O_5 \cdot C_5H_4N_5$ and cannot be a decomposition product of ordinary yeast nucleic acid.

Again, Funk [1912] has prepared from yeast a pyrimidine derivative which cures the polyneuritis in birds induced by a diet of polished rice. The substance is evidently identical with a curative agent which Funk [1912] had previously found in rice polishings and which has the formula $C_{17}H_{20}N_2O_7$. Its physiological properties are considerably different from those of any well-known derivative of yeast nucleic acid (Funk [1913]).

he supposed had the formula $C_{10}H_{12}N_4O_{11}Ba$, and he believed inosinic acid composed of acetic acid, oxalic acid and urea. After a number of unsuccessful attempts by various experimenters to repeat Liebig's work, Hauser [1895] succeeded in getting possession of the substance, and to his surprise found that it contained phosphorus which Liebig had evidently overlooked. Hauser's analysis led to the formula $C_{10}H_{13}N_4PO_8$, and by acid hydrolysis of the substance he obtained phosphoric acid, hypoxanthine and a compound which he took to be trioxysuccinic acid, $C_4H_6(OH)_3CO_2H$ or $C_5H_{10}O_5$. Hauser also found that by mild hydrolysis hypoxanthine is removed from inosinic acid leaving a compound of phosphoric acid with the supposed trioxysuccinic acid.

It was afterwards found independently by Bauer [1907] and by Neuberg and Brahn [1907] : [1908] that the substance which Hauser had taken to be trioxysuccinic acid is pentose, and that by acid hydrolysis, inosinic acid yields chemically equivalent quantities of phosphoric acid, pentose and hypoxanthine according to the equation



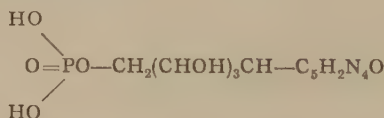
On what they considered sufficient grounds, Neuberg and Brahn concluded that the order of the three groups of inosinic acid is

pentose—phosphoric acid—hypoxanthine

while Bauer believed the arrangement to be

phosphoric acid—pentose—hypoxanthine

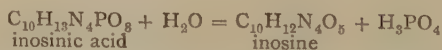
and assigned the formula



In the meantime Hauser and Wenzel [1908] found that the substance obtainable from meat extract called carmine could be separated into hypoxanthine and a new substance having the formula $C_{10}H_{12}N_4O_5$ which by hydrolysis produced pentose and hypoxanthine :—

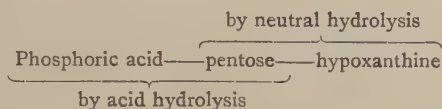


Hauser and Wenzel of course saw that the relation between their new compound "inosine" and inosinic acid is simply that expressed by the equation :—



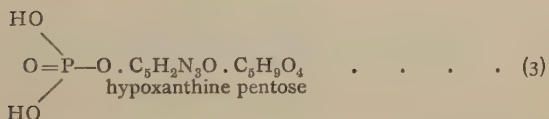
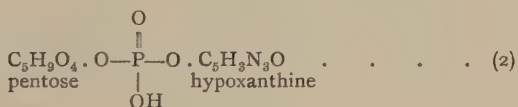
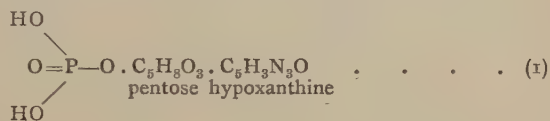
but they curiously concluded that inosine and inosinic acid are formed in muscle *from a common precursor*.

Levene and Jacobs [1909, 1], however, found that one of the substances can be produced from the other. By neutral hydrolysis under pressure inosinic acid was decomposed into phosphoric acid and inosine, identical with the substance obtained from carnine. This reaction taken in connexion with Hauser's previous results proves the order of the groups in inosinic acid¹ :—



As a matter of fact Hauser might have stated the correct structure of inosinic acid in 1905 (except for the matter of trioxysuccinic acid) by making use of an argument that is now employed in connexion with other compounds of this class.

The three groups of inosinic acid can be arranged in three, and only three different orders. That is to say, any one may be the central group connecting the other two :—



Hauser knew that inosinic acid is a dibasic acid so that formula (2) is excluded. He found also that hypoxanthine is removed from inosinic acid more easily than is phosphoric acid. This excludes formula (3), leaving formula (1).

Inosinic acid belongs to a class called "nucleotides" which may be defined as compounds in which a carbohydrate group links a phosphoric acid group with a purine or a pyrimidine group. It will be shown that yeast nucleic acid is also a nucleotide.

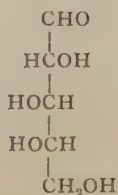
The carbohydrate of inosinic acid (or inosine) is the common pentose of the animal body and will be considered in detail.

¹ See also Levene and Jacobs [1908].

The Carbohydrate Group of Yeast Nucleic Acid. (d-Ribose.)

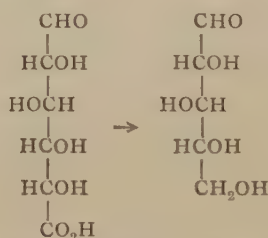
According to the theory eight aldopentoses are possible, of which six were known at the time that inosine-pentose was under examination.

1. l-Arabinose, a natural plant pentose :—



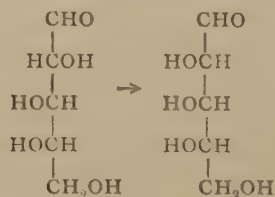
2. l-Xylose, a natural plant pentose, supposed to be the pentose obtainable from the β -nucleoprotein of the pancreas (Neuberg [1902]) and the liver (Wohlgemuth [1900]).¹

Salkowski and Neuberg [1902] had obtained the substance by putrefaction of d-glucuronic acid :—



and it seemed *a priori* that inosine-pentose would be found identical with this sugar.

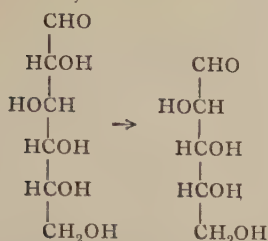
3. l-Ribose, prepared from l-arabinose through arabonic acid by the pyridine rearrangement (Fischer and Piloty [1891]):—



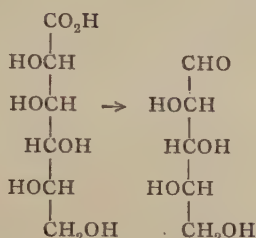
4. l-Lyxose, unknown.

5. d-Arabinose, prepared from d-glucose by degradation (Wohl [1893]):—

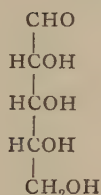
¹ For identification of pentose and its occurrence in the body, see Salkowski [1899], Neuberg [1904].



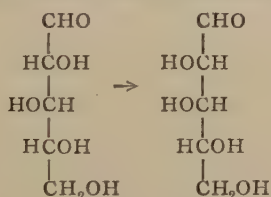
6. d-Xylose, prepared from d-gulonic acid by degradation (Fischer and Ruff [1900]):—



7. d-Ribose, unknown. Its configuration follows from that of l-ribose:—

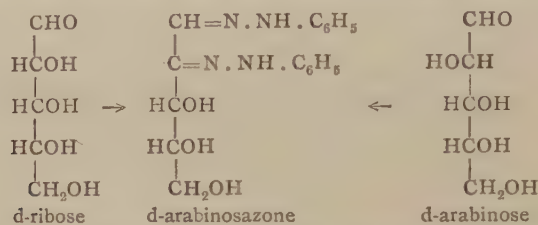


8. d-Lyxose, prepared from l-xylose through xylonic acid by the pyridine rearrangement (Fischer and Bromberg [1896]):—

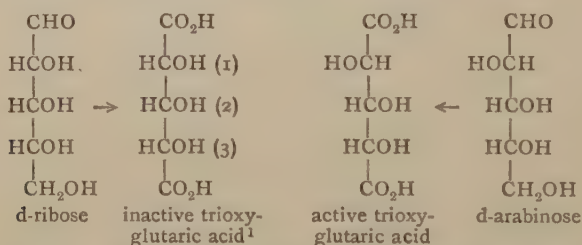


The identification of inosine-pentose with one of these substances might have been expected to offer little difficulty, but as a matter of fact four different conclusions were reached in the four laboratories where the pentose was under examination. Upon evidence considered sufficient by the various writers, inosine-pentose was believed to be l-xylose by Neuberg and Brahn [1908], dl-arabinose by Bauer [1907], and d-lyxose by Haiser and Wenzel [1909]. Levene and Jacobs [1908] found reasons for questioning the validity of all these suppositions and called the sugar "carnose". While the matter was

under spirited discussion, Levene and Jacobs [1909, 2] prepared the pentose from carnine in beautifully crystalline form, and proved that its structure corresponds to the space configuration that had been assigned to the hypothetical d-ribose. With the crystalline sugar in hand its structure could be determined without great difficulty. Its lævo-rotation excluded four of the eight isomers. It was found to yield an osazone identical with d-arabinosazone, thus excluding both lyxose and xylose, and as the sugar could be proven an *aldo* pentose one is left a choice between the two pentoses that form d-arabinosazone :—



Finally carnose was found to yield, by oxidation, *inactive* trioxo-glutaric acid (Levene and Jacobs [1909, 7]), and d-arabinose became excluded :—



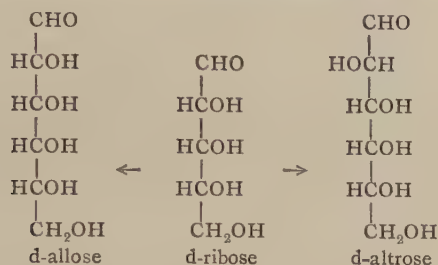
While carnose was proven by exclusion to be d-ribose, a more direct demonstration is available. Van Eckenstein and Blanksma [1909] obtained l-ribose in crystalline form and found its melting-point 87° and its specific rotation $+18.8^\circ$. As carnose melts at the same temperature and has a specific rotation arithmetically equal to that of l-ribose but of opposite sign, the two pentoses must be antipodal.

Haiser and Wenzel concede without reservation [1910] that the matters treated are sufficient to establish the identity of carnose with d-ribose; nevertheless, Levene and Jacobs [1911, 1] have tested the

¹ Trioxo-glutaric acid is possibly not inactive but its activity is so slight as to make it appear so. In so far as the arrangements of the groups around carbon atoms *one* and *three* are concerned, the substance should be inactive by intramolecular compensation. But carbon atom *two* connects two groups that are oppositely arranged in an optical sense and must therefore be asymmetric. The subject has been much discussed.

l-Xylose also produces an inactive trioxo-glutaric acid but is sufficiently excluded on other grounds,

question by an examination of a large number of derivatives and have found their first conclusion overwhelmingly sustained. Among other derivatives of d-ribose they prepared two of the missing aldo-hexoses [1910, 2]. By the ordinary reaction with hydrocyanic acid "d-allose" and "d-altrose" were produced and separated from one another:—



Up to the time of Levene and Jacob's work d-ribose had not been found in nature; but as will appear it is the only pentose of the animal body (Levene and Jacobs [1910, 3]: [1909, 3]) and is also the pentose of plant nucleic acid so that its physiological distribution is very wide. The corresponding alcohol, adonite, is the only natural pentite.

Guanylic Acid.

In the year 1894 appeared Hammarsten's well-known contribution on the nucleoproteins of the pancreas [1894]. He described two substances, α -nucleoprotein and β -nucleoprotein, one of which he believed to be formed from the other by decomposition at the boiling-point. But this idea though a natural conclusion from the facts in Hammarsten's possession does not represent the true state of the case for the two nucleoproteins are quite independent of one another. The β -nucleoprotein may be removed from the tissue with boiling water while the α -nucleoprotein remains with the coagulum and there is no evidence to show that β -nucleoprotein is a constituent of the cell nucleus.

Hammarsten gave his attention exclusively to the β -compound. He found that it contained phosphorus, but produced only one of the amino-purines (guanine). Among its hydrolytic products was obtained a substance which responded to the phloroglucine test (Tollens [1896]), reduced Fehling's solution, produced furfural and formed an osazone melting at 159°. From these facts Hammarsten concluded with some reserve (see Mayer [1900]) that he was dealing with a pentose. He also found that the nucleoprotein can be decomposed into its protein and non-protein constituents by the action of warm alkalis,

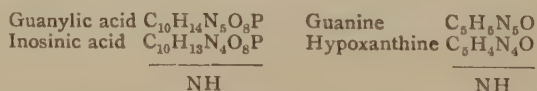
and gave over the non-protein component (guanylic acid) to his pupil Bang for a chemical examination. Bang [1898-9]: [1900-1]: [1901] found that guanylic acid produces phosphoric acid, guanine and pentose, but neither adenine nor thymine. In addition he obtained from guanylic acid what he thought to be glycerophosphoric acid and entered into a discussion of a relation of nucleic acid to lecithin.

Subsequently Bang and Raachou [1904] described a more convenient method of preparing guanylic acid from the pancreas, and replaced the former structural formula by a new one. They confirmed the previous finding of glycerine. But the repetition of this work by v. Fürth and Jerusalem [1907] led to an ordinary nucleic acid which produced both guanine and adenine.

Guanylic acid was fast coming to be regarded as mythical when Steudel [1907, 3] showed that by Hammarsten's original method a substance can be obtained from the pancreas which yields phosphoric acid, guanine and pentose but neither glycerine nor any other product. Identical or very similar guanylic acids have since been found widely distributed in animal glands (Jones and Rowntree [1908]; Levene and Mandel [1908, 2]).¹

More recently Levene and Jacobs [1912, 3] prepared a crystalline brucine salt of guanylic acid whose analysis shows that the free acid has the formula $C_{10}H_{14}N_5PO_8$ and not the more complicated formula upon which Bang insists, and Knopf [1914] has found that guanylic acid may be converted into xanthylic acid by deamination with nitrous acid:—

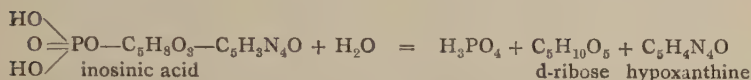
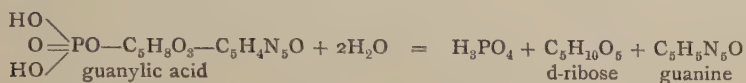
So far as concerns empirical composition, therefore, guanylic acid and inosinic acid bear to one another the relation of guanine to hypoxanthine:—



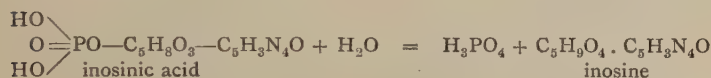
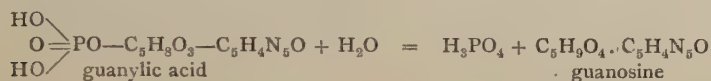
Mindful of their results with inosinic acid Levene and Jacobs [1909, 4] submitted guanylic acid to neutral hydrolysis at 175° under pressure, and obtained a substance which crystallized out of hot water in needles of the composition $C_{10}H_{13}N_5O_5 \cdot 2H_2O$ and decomposed by

¹ Guanylic acid was known to the earlier workers only in the form of an impure potassium salt thrown out of aqueous solution by the addition of alcohol. But Jones and Richards [1914] found that by means of its lead salt, free guanylic acid may be obtained in pure form, soluble in hot water and deposited from the hot solution as it cools. Read [1917] also prepared free guanylic acid in this way and purified it by deposition from hot water. Levene [1919, 2] has finally succeeded in getting the preparation of Jones and Richards in crystalline form.

acid hydrolysis into guanine and d-ribose. Thus guanylic acid and inosinic acid are similarly constituted mononucleotides consisting of phosphoric acid and purine base united by d-ribose. Upon hydrolysis with boiling mineral acid they are decomposed into their three constituents :—



By neutral hydrolysis under pressure, phosphoric acid is removed and the nucleosides, guanosine and inosine, are formed :—



From a structural point of view these two mononucleotides may be looked upon as simple nucleic acids. Their groups are those of *plant* nucleic acid, and they cannot therefore be directly derived from the nucleic acid of the *animal* tissues in which they occur.

Schulze and Bosshard long ago obtained from plant tissues a substance which they called vernine [1886], which, after the work of Levene and Jacobs appeared, Schulze found to be guanine-pentoside yielding a lævo-rotatory pentose [1910]. Schulze and Trier [1910] afterwards compared vernine with guanosine obtained from Levene and found the two substances identical. Steudel and Brigl's [1910] inability to prepare guanosine from guanylic acid is therefore without particular significance.¹

The Nucleosides of Yeast Nucleic Acid.

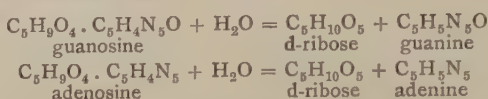
Their results with inosinic acid and guanylic acid naturally led Levene and Jacobs to employ similar methods in the study of yeast nucleic acid. The substance was submitted to neutral hydrolysis at 175° under pressure when decomposition occurred with the formation of four crystalline nucleosides :—

¹ Guanosine is so commonly met in studies of yeast nucleic acid fermentation as to be an annoyance. It can be prepared from yeast nucleic acid without the slightest difficulty (see Schittenhelm and Wiener [1912], Amberg and Jones [1913], Tsuji [1913], Jones and Richards [1914]).

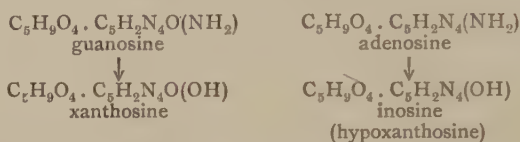
NUCLEIC ACIDS

Guanosine	$C_{10}H_{13}N_5O_5$	[1909, 5].
Adenosine	$C_{10}H_{13}N_5O_4$	[1909, 6].
Cytidine	$C_9H_{13}N_3O_5$	[1910, 4].
Uridine	$C_9H_{12}N_2O_5$	[1910, 4].

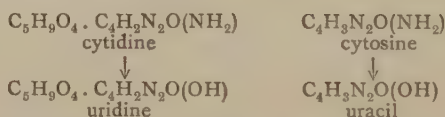
Guanosine was found identical with the nucleoside formed by neutral hydrolysis of guanylic acid and of course is decomposed by boiling mineral acid into guanine and d-ribose. Correspondingly adenosine forms adenine and d-ribose :—



The two amino-purine-nucleosides, adenosine and guanosine, are converted into the corresponding oxy-purine nucleosides, inosine and xanthosine, by deamination with nitrous acid, just as the free amino-purines are converted into the free oxy-purines :—



In like manner cytidine, an amino-pyrimidine-nucleoside, can be converted into uridine, its corresponding oxy-pyrimidine-nucleoside, just as cytosine forms uracil :—



The two pyrimidine-nucleosides are very resistant to hydrolytic agents and cannot be decomposed into their two components, but certain of their derivatives are easily hydrolysed forming the corresponding free pyrimidine derivatives thus :—

hydro-uridine gives hydro-uracil.

hydro-cytidine gives hydro-cytosine.

nitro-uridine gives nitro-uracil (Levene and La Forge [1911]).

The purine nucleosides do not reduce Fehling's solution, and their two constituents are therefore joined in glucosidal linkage. Nor do the pyrimidine nucleosides reduce Fehling's solution, but their resistance to hydrolytic agents is such as to suggest a different kind of linkage from that of the purine nucleosides. These and other questions concerning the finer points of structure are discussed by Levene and Jacobs but need not be taken up here.

The Nucleotides of Yeast Nucleic Acid.

In the preceding section the following matters were noted :—

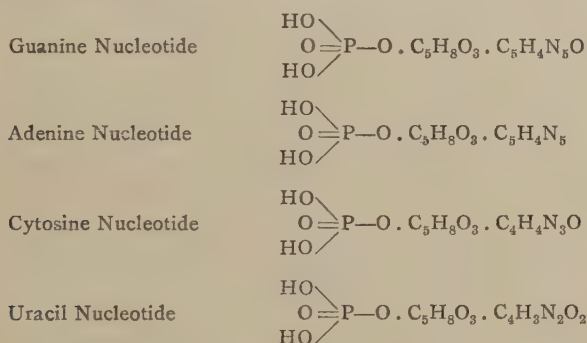
1. Inosinic acid and guanylic acid have the nucleotide structure.

Phosphoric acid—carbohydrate—base.

2. By hydrolysis with mineral acid each of these nucleotides is resolved into its three constituents. Yeast nucleic acid behaves in the same way.

3. By hydrolysis with ammonia, inosinic acid and guanylic acid both characteristically lose phosphoric acid and form nucleosides. So does yeast nucleic acid. It forms four nucleosides one of which is identical with the nucleoside produced from guanylic acid.

From these considerations, Levene and Jacobs were led to assume that yeast nucleic acid is a tetra-nucleotide composed of the groups of four mono-nucleotides :—



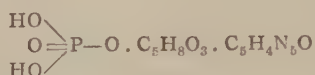
This is the nucleotide theory of the constitution of yeast nucleic acid and could logically proceed only from the four nucleosides, a matter which should be kept in mind when the constitution of *thymus* nucleic is taken up; because the four analogous nucleosides of thymus nucleic acid are not known.

The theory suggested the possibility of preparing four mono-nucleotides from yeast nucleic acid. All four of the nucleotides have been prepared.

Guanine Nucleotide.—This substance was first prepared from yeast nucleic acid by the action of an aqueous extract of pig's pancreas (Jones [1911, 1]) and afterwards by the action of the ferments of yeast (Jones and Richards [1914]). The nucleotide was finally obtained almost quantitatively from yeast nucleic acid by hydrolysis with ammonia at a low temperature (105°-115°) (Jones and Germann [1916], Jones and Read [1917]: [1917, 4]).

It is deposited in amorphous granules from a hot concentrated

aqueous solution as it cools, and analysis of the material thus obtained leads sharply to the formula $C_{10}H_{14}O_3N_5P$. By hydrolysis with ammonia at 145° it produces guanosine and forms a crystalline brucine salt, $C_{10}H_{14}O_3N_5P(C_{23}H_{26}N_2O_4)_2 \cdot 7H_2O$, which melts at 205° . The nucleotide is therefore a dibasic acid; and as it sets free guanine much faster than phosphoric acid its groups must be arranged as indicated in the formula ¹



An exhaustive comparison of guanine nucleotide with guanylic acid showed that the two substances do not differ from one another.

This identity suggests the explanation of an interesting and curious matter. The nucleotide which is a constituent of animal cell nuclei, i.e. the so-called animal nucleic acid, contains a hexose group. This can scarcely be doubted, for by severe acid hydrolysis the substance yields both formic acid and lævulinic acid, and by oxidation with nitric acid it forms one of the saccharic acids. On the other hand, the tetra-nucleotide of plant cell nuclei or plant nucleic acid possesses a pentose group, and pentose is formed when the substance is hydrolyzed. Guanylic acid is a mono-nucleotide present in animal glands and might be expected to resemble in its chemical structure the nucleotide of animal tissues. But this is not the case. Guanylic acid contains a pentose group, not a hexose group, and by acid hydrolysis it forms not only a pentose but the particular pentose which is formed similarly from plant nucleic acid (d-ribose). The idea is at once suggested that the guanylic acid of *animal* tissues is formed from the nucleic acid of *plant* food, and this idea is strongly supported by the actual production of guanylic acid from yeast nucleic acid by the action of ferments.

In order to throw additional light upon this question, Jones and Richards [1915] undertook the quantitative preparation of guanylic acid from the pancreas of different animal species, the ox and the pig. The substance cannot be obtained in very great amount from the pancreas of either species, and the methods in use for its isolation are far from what might be desired; so that a slight difference observed would be without significance. But they obtained about three and one-half times as much guanylic acid from ox pancreas as from an equal weight of pig pancreas.

All things considered, one is inclined to believe that the guanylic

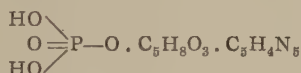
¹ See under "Inosinic Acid".

acid of animal tissues has no direct relation to animal nucleic acid, but owes its origin entirely to the nucleic acid of plant food.

Adenine Nucleotide.—Jones and Kennedy [1918] found that when a neutral or faintly alkaline solution of nucleic acid is treated with potassium permanganate, its various groups are destroyed in a definite order, leaving an undecomposed residue of the nucleotide, whose chemical isolation offers little difficulty; and the extent to which this destruction of the nucleic acid will proceed can be closely controlled by the amount of permanganate used. From the products of such a partial oxidation, Jones and Kennedy isolated adenine nucleotide as macroscopic crystalline needles having the composition $C_{10}H_{14}O_7N_5P \cdot H_2O$. By acid hydrolysis it forms phosphoric acid and adenine in the amounts required by the formula but produces neither guanine, cytosine nor uracil. It forms a crystalline brucine salt



which melts at 177° – 178° . As the nucleotide is a dibasic acid and sets free its adenine very much faster than its phosphoric acid, the arrangement of its groups must be in the order



Cytosine Nucleotide.—Thannhauser and Dorfmueller [1915] decomposed yeast nucleic acid into its four nucleotides by heating with ammonia in an autoclave at 122° , and treated the product with a large amount of alcohol which precipitated three of the nucleotides (adenine-, guanine-, cytosine-), and left the fourth (uracil-) in solution. This mixture of the three nucleotides was boiled with dilute acid for the destruction of the two purine nucleotides, so that the resistant cytosine nucleotide which survived could easily be isolated in pure crystalline form [1918], [1919]. Thannhauser's work is involved in what he supposed to be a tri-nucleotide, and seems at first glance to be out of harmony with the well-established work of others. But there is no contradiction, as will be explained in the next section. Cytosine nucleotide was the first crystalline nucleotide prepared from yeast nucleic acid.¹

Uracil Nucleotide.—Levene [1918, 1] discovered the characteristic insolubility of the brucine salt of uracil nucleotide in 35 per cent. alcohol. This enabled him [1920, 2] to isolate crystalline uracil nucleotide from the brucine salt mixtures that had been prepared by

¹ Crystalline cytosine nucleotide preceded the crystalline adenine nucleotide of Jones and Kennedy by about six months.

Jones and Read [1917, 1]: [1917, 3]. The matter will be discussed in the next section. Uracil nucleotide is so soluble that its isolation is somewhat difficult.

On Compound Nucleotides.

Without a single exception, every modern investigator in this field has prepared from yeast nucleic acid a substance which he believed to be a chemical individual containing more than one nucleotide group. (Levene [1917, 1]; Jones and Richards [1915]; Thannhauser [1914]; Thannhauser-Dorfmueller [1915]; Jones and Germann [1916]; Jones and Read [1917, 1], [1917, 3]; Bottomley [1917].) But all of these supposed compound nucleotides are mechanical mixtures of mono-nucleotides. It was to be expected that the isolation of the individual nucleotides would offer considerable difficulty, and it is not surprising that the earlier researches were attended by some confusion. However, it is now possible to reconcile all apparent contradictions, and it is profitable to do so because the discussion will disclose the most important properties of the nucleotides.

Cytosine-Uracil Di-nucleotide.—Nine years ago, Levene and Jacobs [1911, 2] boiled yeast nucleic acid with dilute mineral acid for the destruction of the purine nucleotide groups. After removal of the destruction products, the surviving pyrimidine nucleotides were converted into an amorphous mixture of barium salts, and these in turn to a mixture of amorphous sodium salts. Levene and Jacobs did not pretend to have isolated the individual pyrimidine nucleotides, nor even to have prepared a pure mixture of the two, but stated frankly that one of their salts contained 36 per cent. of ash, and that the other gave less satisfactory analytical data the more its purification was attempted.

Six years later, Jones and Read [1917, 3] decomposed yeast nucleic acid in a similar way, but they made lead salts of the surviving nucleotides, from which the free nucleotides could easily be prepared. The free nucleotides were found to yield beautifully crystalline brucine salts which could be purified to any desired extent by recrystallization from hot water. The appearance, constancy of melting-point and chemical composition of this brucine preparation led Jones and Read to suppose that they were not dealing with a mixture of two mono-nucleotides, but with a di-nucleotide.

Levene [1917, 1] afterwards repeated and verified the experimental work of Jones and Read. He concluded that the substance is cytosine-uracil di-nucleotide, and even found from amino nitrogen

determinations that the cytosine and uracil groups were present in equivalent quantities, as should be the case with a di-nucleotide.

Subsequently, Levene [1918, 1] made an analytical discovery which has found general application in dealing with nucleotide mixtures (Levene [1918, 2], [1919, 1, 2, 3, 4], [1920, 1, 2]; Jones and Abt [1920]). He recrystallized the brucine salt of the supposed cytosine-uracil di-nucleotide nine times from 35 per cent. alcohol, and found that the final crystals consisted of the pure brucine salt of uracil nucleotide. From the brucine salt a crystalline barium salt was prepared and analysed. By evaporation of the nine mother liquors from the above brucine salt, the impure brucine salt of cytosine nucleotide was obtained. This was likewise converted into a crystalline barium salt which was analysed¹ [1919, 1].

Therefore, cytosine-uracil di-nucleotide as a chemical individual disappears. But it served a useful purpose: it gave the characteristic slow rate at which pyrimidine nucleotides set free their phosphoric acid by acid hydrolysis—a value that will be used in discussing the nucleotide linkages of yeast nucleic acid (p. 45).

Adenine-Uracil Di-Nucleotide and Adenine-Cytosine-Guanine Tri-Nucleotide.—The literature on this subject is very difficult to follow, but can be easily disentangled if one has in mind the “key”.

When yeast nucleic acid undergoes hydrolysis either by the action of certain tissue extracts² or by the action of ammonia at low temperatures³ (100°–125°), the substance is decomposed into its four constituent mono-nucleotides. If one will treat the latter product (alkaline as it is) with successive portions of alcohol, the ammonium salts will be precipitated in a somewhat definite order:—

first	guanine nucleotide
second	cytosine nucleotide
third	adenine nucleotide
last	uracil nucleotide

Three cases are of special interest:—

I. When “the aqueous ammoniacal fluid” is treated with an equal volume of alcohol, only guanine nucleotide is precipitated, and the other three remain in solution with a trace of guanine nucleotide—

¹ Thannhauser and Dorf Müller's [1918] preparation of free crystalline cytosine nucleotide preceded by a year Levene's preparation of its barium salt. But Thannhauser's results were not known by Levene nor by anyone outside of Germany.

² Pancreatic juice (Thannhauser [1914]), boiled pancreatic extract (Jones [1920, 2]), or pancreatic extract that has been kept a long time at 40° (Jones and Richards [1915]).

³ Thannhauser and Dorf Müller [1915]; Jones and Germann [1916].

guanine nucleotide

.

cytosine nucleotide

adenine nucleotide

uracil nucleotide

This is the method used by Jones and Read [1917, 1] for the preparation of pure guanine nucleotide,¹ and is the one referred to by Jones and Germann [1916]. Jones and Read called their soluble fraction adenine-uracil di-nucleotide because analysis of a crystalline brucine salt of the mixture gave values exactly intermediate between those required for adenine nucleotide and uracil nucleotide. Levene [1918, 2] afterwards confirmed their analytical finding. This is easy to understand. Cytosine nucleotide acts like a buffer, since its chemical composition is almost exactly that of adenine-uracil di-nucleotide.

Levene crystallized the brucine salt of the supposed adenine-uracil di-nucleotide nine times out of 35 per cent. alcohol, and found that the last crystals were the practically pure brucine salt of uracil nucleotide. Later both Levene [1919, 4] and Jones and Abt [1920] prepared from the 35 per cent. alcoholic mother liquors pure adenine nucleotide, identical with the crystalline substance that Jones and Kennedy [1918]: [1919] had previously described. Finally Levene [1920, 2] showed that the supposed adenine-uracil di-nucleotide contains also cytosine-nucleotide.²

II. When the "aqueous ammoniacal fluid" is treated with more than an equal volume of alcohol, a well-defined granular precipitate settles quickly, leaving a perfectly transparent supernatant liquid. This continues to be the case until about $1\frac{3}{4}$ volumes of alcohol have been added, when an opalescence rather suddenly appears (Jones and Germann [1916]). This phenomenon indicates that the precipitation of adenine nucleotide has just begun. If one will cease adding alcohol just at or just before this point he will make a sharp separation of the nucleotides into two fractions:—

¹ See also Read [1917].

² This can easily be shown. By evaporation of the mother liquor from adenine nucleotide (Jones and Abt [1920, p. 577]) prisms of cytosine nucleotide are deposited. These may be gotten perfectly pure by recrystallization from hot water (Jones and Abt [not published]).

guanine nucleotide
 cytosine nucleotide

 adenine nucleotide
 uracil nucleotide

This is the method used by Jones and Germann [1916], and probably also by Jones and Richards [1915]. It accounts for the expressions adenine-uracil di-nucleotide and guanine-cytosine di-nucleotide. Jones and Germann found that their soluble fraction was an equal mixture of the two nucleotides. It gave the required amount of adenine, and the distribution of its phosphorus showed that it contained its purine and pyrimidine groups in exactly equivalent quantities¹ [1916, p. 100].

It is reasonably certain that the application of the method described for the separation of mono-nucleotides from one another would show that both preparations of Jones and Germann are mechanical mixtures.

Bottomley [1917] has described di-nucleotides obtained from peat. His work may be correct, but cannot be accepted as final until his preparations have been submitted to the test applied to other supposed compound nucleotides.

III. When the "aqueous ammoniacal fluid" is treated with a large excess of alcohol (4 volumes), three of the nucleotides are precipitated, and one is left in solution :—

guanine nucleotide
 cytosine nucleotide
 adenine nucleotide

 uracil nucleotide

This is the method used by Thannhauser and Dorfmueller [1915].

Thannhauser and Dorfmueller prepared a crystalline brucine salt of the three precipitated nucleotides and called the substance a tri-nucleotide. From the supposed reality of the tri-nucleotide, Thannhauser [1917] proposed for yeast nucleic acid a complicated structural formula which involves seven nucleotide groups.

There is no doubt that the substance which Thannhauser and Dorfmueller obtained by heating yeast nucleic acid with ammonia is identical with the mixtures that both Jones and Read and Levene obtained by the same process. The two latter were shown to be a mixture of the four mono-nucleotides.

¹ The analytical data were given afterward in an article by Jones and Read [1917, 2].

The Partition of Phosphorus in Yeast Nucleic Acid.

The nucleotide structure of yeast nucleic acid indicates the paths along which the substance may be expected to undergo decomposition whether by chemical means or under the influence of physiological agents. Such a decomposition could produce a variety of substances: tri-nucleotides, di-nucleotides, mono-nucleotides, nucleosides, pyrimidine derivatives, purine derivatives, pentose, and phosphoric acid. So that, while the hydrolytic decomposition of nucleic acid is not a difficult matter to discuss, its experimental investigation is almost impossible; for of the decomposition products enumerated, only one (phosphoric acid) admits of exact quantitative determination. The chief difficulty lies in the estimation of the pyrimidine derivatives. But since the *purine derivatives* can be estimated with a fair degree of accuracy, the principal obstruction to investigation in this field would be removed if one could devise a precise experimental method of determining the ratio of the number of combined purine groups to the number of combined pyrimidine groups. From this point of view the following experiments are of interest (Jones [1916, 1]).

Ten portions of commercial yeast nucleic acid from the same specimen were weighed into appropriate vessels, and in one of these the total phosphoric acid was determined. The other nine portions were submitted to hydrolysis with 5 per cent. sulphuric acid at 100° for various periods from $\frac{1}{4}$ hour to $7\frac{1}{2}$ hours, and after removal of the guanine with ammonia, the phosphoric acid was precipitated with magnesia mixture and weighed as magnesium ammonium phosphate.¹ The weight of magnesium ammonium phosphate obtained in each experiment was divided by the weight of nucleic acid from which it was produced, and from the comparable results so obtained (Table I, Column 4) the upper curve in Fig. 1 was constructed. (The method of construction is so obvious as to require no explanation.)

This curve admits of an easy interpretation. Its rapid initial rise, sharp turn, and termination in a straight line slightly inclined to the axis, show clearly that in the acid hydrolysis of yeast nucleic acid, phosphoric acid is progressively liberated from two sources. From the one, the production is rapid and complete in less than two hours; from the other, it is much slower, continues beyond the time consumed in these experiments, and is so regular that its amount is definitely seen to be equivalent to 10 mg. of magnesium ammonium phosphate per hour. If we subtract this amount from the figures of Column 4,

¹ Magnesium ammonium phosphate is a proper form in which to weigh phosphoric acid [Jones, 1916, 2].

TABLE I.—HYDROLYSIS OF COMMERCIAL YEAST NUCLEIC ACID WITH 5 PER CENT. SULPHURIC ACID 20 C.C. PER GM.

Nucleic Acid.	Duration of Hydrolysis.	Magnesium Ammonium Phosphate $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$.			
		Obtained.	Per gm. of Nucleic Acid.	Pyrimidine Correction.	From Purine Nucleotides.
gm.	hrs.	gm.	gm.	gm.	gm.
1'1782	$\frac{1}{4}$	0'1060	0'090	0'003	0'087
0'7347	$\frac{1}{2}$	0'1284	0'175	0'005	0'170
0'8644	1	0'2315	0'269	0'010	0'259
0'9837	2	0'3125	0'318	0'020	0'298
0'9251	3	0'3038	0'329	0'030	0'299
1'0305	4	0'3488	0'338	0'040	0'298
0'8333	5	0'2876	0'345	0'050	0'295
0'9927	6	0'3565	0'359	0'060	0'299
0'9667	$7\frac{1}{2}$	0'3625	0'375	0'075	0'300
0'8179	Total.	0'5112	0'590	Half.	0'295

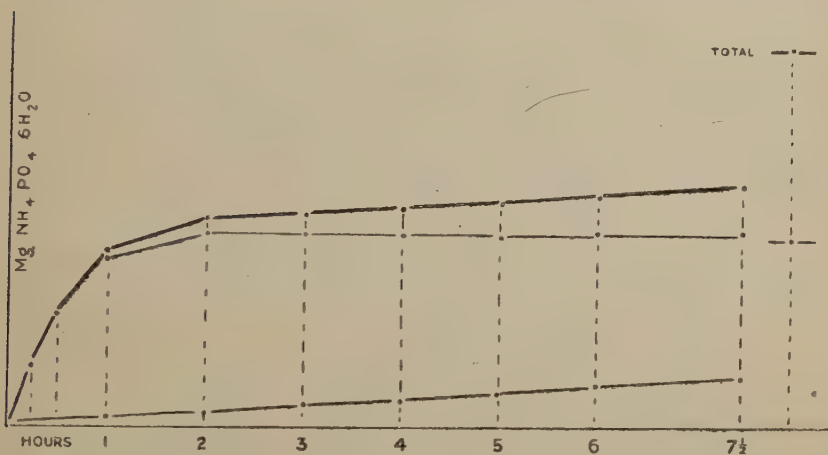


FIG. 1.

Table I, we obtain the figures of Column 6, from which the lower curve of Fig. 1 has been constructed. This curve ends in a straight line, parallel to the axis, whose ordinate corresponds to half the total phosphoric acid obtainable from nucleic acid; or in other words, the phosphoric acid which is rapidly and completely liberated by acid hydrolysis of yeast nucleic acid is half the total.

If space permitted it could easily be shown that at the point where the curve makes its sharp turn, the purines have been completely set free while the pyrimidines are still combined. Moreover, the existing literature forces the conclusion that the two sources of phosphoric acid which we are discussing are the *purine* nucleotides and the *pyrimidine*

nucleotides. As the two sources of phosphoric acid are equal in amount the purine groups of nucleic acid must be equal in number to the pyrimidine groups.

If this view of the matter be accepted the following deductions may be drawn.

A tetra-nucleotide (such as yeast nucleic acid) which contains two purine groups and two pyrimidine groups gives off half of its phosphoric acid by mild hydrolysis with sulphuric acid. So also should a di-nucleotide that contains one purine group and one pyrimidine group. But in such cases it would be easy to show that the di-nucleotide contains but one purine group or even that it produces twice as much of the purine compound as could be obtained from a tetra-nucleotide.

A purine mono-nucleotide should give off all of its phosphoric acid while a pyrimidine mono-nucleotide should give off none of its phosphoric acid by mild acid hydrolysis.

A tri-nucleotide offers an excellent opportunity for the application of this method. If such a compound should contain one pyrimidine group and two purine groups it might be expected to give off two-thirds of its phosphoric acid; but if it should contain two pyrimidine groups and one purine group it would give off only one-third of its phosphoric acid by mild acid hydrolysis.

In case a nucleotide is contaminated with other substances (nucleosides, purine bases, or any compounds that do not produce phosphoric acid) its total phosphorus will be low but the numerical index of the nucleotide will not be affected by the presence of the impurity.

It is naturally interesting to note how the individual nucleotides actually conduct themselves when submitted to hydrolysis with dilute sulphuric acid.

Guanine nucleotide was found to contain no firmly bound phosphoric acid (Jones and Read [1917, 4]). The "easily split" is equal to the "total".

Adenine nucleotide gave a curve that is practically coincident with the lower curve of Fig. 1 (i.e. the hypothetical purine curve) (Jones [1919, 2]).

A mixture of equivalent quantities of adenine nucleotide and uracil nucleotide¹ (Jones and Germann [1916]) gave off half of its phosphoric acid easily and held the other half firmly.

A mixture of cytosine nucleotide and uracil nucleotide² (Jones and Read [1917, 3]) was found to give off an amount of phosphoric acid

¹ Originally supposed to be a purine-pyrimidine di-nucleotide.

² Originally thought to be a pyrimidine di-nucleotide.

that corresponds to only 10 mg. of magnesium ammonium phosphate per hour in accordance with the inclined straight line at the bottom of Fig. 1.

The Nucleotide Linkages of Yeast Nucleic Acid

The earliest investigations of nucleic acid had shown that by mild acid hydrolysis, the purines are set free with part of the phosphoric acid and part of the carbohydrate; but that violent hydrolytic processes are required to set free the pyrimidines with the remainder of the phosphoric acid and carbohydrate. A great amount of ingenuity was therefore not required to formulate a hypothetical structure for nucleic acid. The substance should be composed of four complexes (two purine complexes and two pyrimidine complexes), all of which contain a group of phosphoric acid and a group of carbohydrate, but each complex contains a different one of the four nitrogenous groups.

Change the word "complex" to the word "nucleotide" and the above becomes essentially the modern nucleotide theory of the constitution of yeast nucleic acid.

But one important matter was not touched upon by the early investigators. At what points are the nucleotides joined together to form yeast nucleic acid or, in other words, what is the mode of nucleotide linkage in yeast nucleic acid? Without any experimental evidence, it was finally agreed that the nucleotide linkage is through the phosphoric acid groups, and this assumption remained undisturbed until Jones, Germann and Read furnished the experimental evidence to show that this mode of nucleotide linkage is not correct. The following is an independent confirmation and an extension of this evidence.

I.—A comparison of the rate at which phosphoric acid is set free from yeast nucleic acid with the rate at which phosphoric acid is set free from the individual nucleotides (Jones [1920, 1]).

After an extended study of the rate at which phosphoric acid is split from yeast nucleic acid by hydrolysis with mineral acid, Jones and Riley (see Jones [1916]) concluded that the nucleic acid liberates its phosphoric acid by two widely different laws: and they predicted that if the individual nucleotides were ever prepared from yeast nucleic acid and examined in this respect, each of the purine nucleotides would be found to obey one of these laws, and each of the pyrimidine nucleotides would obey the other. Various preparations of mixed nucleotides were afterwards obtained, and their conduct justified this assumption, since each nucleotide preparation was found to set free its phosphoric acid in accordance with the proportion of purine and

pyrimidine material that it contained.¹ Finally Jones and Kennedy [1918]: [1919] found that the nucleotide groups are burned from yeast nucleic acid by potassium permanganate in a definite order, adenine nucleotide being the last to go: so that it was possible to obtain pure adenine nucleotide in this way.

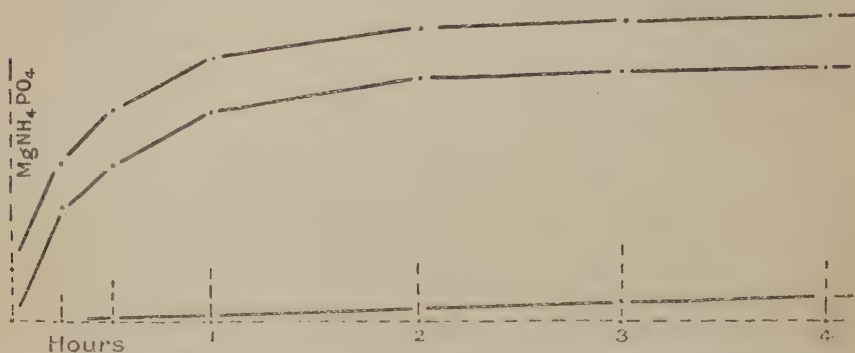


FIG. 1.—The two curves at the top show the rate at which phosphoric acid is set free from the purine nucleotides. The straight line shows the rate for the pyrimidine nucleotides.

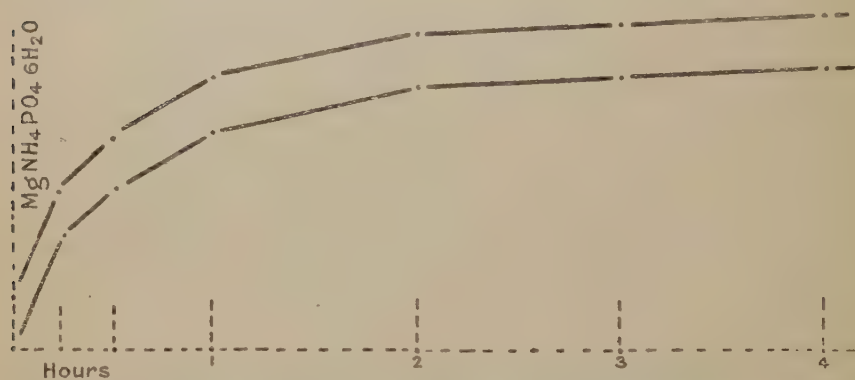


FIG. 2.—The upper curve is a fusion of 3 with 1 or 2. The lower curve shows the rate at which phosphoric acid is set free from yeast nucleic acid.

The substance consists of characteristic long transparent needles of the composition $\text{C}_{10}\text{H}_{14}\text{N}_5\text{PO}_7 \cdot \text{H}_2\text{O}$ and its possession furnished an excellent opportunity to ascertain the rate at which phosphoric acid is set free from a purine nucleotide. The rate was found to be the same as that of the previously determined rate for guanine nucleotide (Jones and Read [1917, 4]), both being the rate that Jones and Riley had predicted for purine nucleotides.

This relation is expressed diagrammatically in Figs. 1 and 2, which are constructed with the same system of co-ordinates and the

¹ Jones and Germann [1916]. For analytical data see Jones and Read [1917, 2].

same linear units. Abscissæ represent time and ordinates represent weights of phosphoric acid (expressed in terms of ammonium magnesium phosphate). In order to avoid confusion, the curves in each diagram have different origins placed vertically above one another.

The upper curve of Fig. 1 was constructed from experimental data obtained with guanine nucleotide three years ago by Jones and Read [1917, 4].

The lower curve of Fig. 1 was similarly constructed from experimental data obtained more recently by Jones and Kennedy [1918]: [1919] with adenine nucleotide.

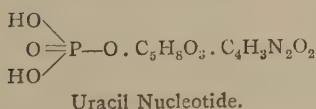
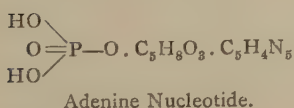
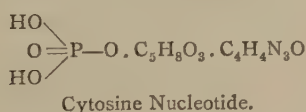
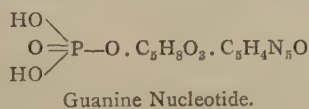
The two curves are practically identical, and may be called the law for purine nucleotides.

The straight line of Fig. 1 was drawn from experimental data obtained by Jones and Read [1917, 3] with a mixture of the two pyrimidine nucleotides. It shows the very slow regular rate which may be called the law for pyrimidine nucleotides.

The upper curve of Fig. 2 was constructed by fusing the pyrimidine curve with the purine curve of Fig. 1.

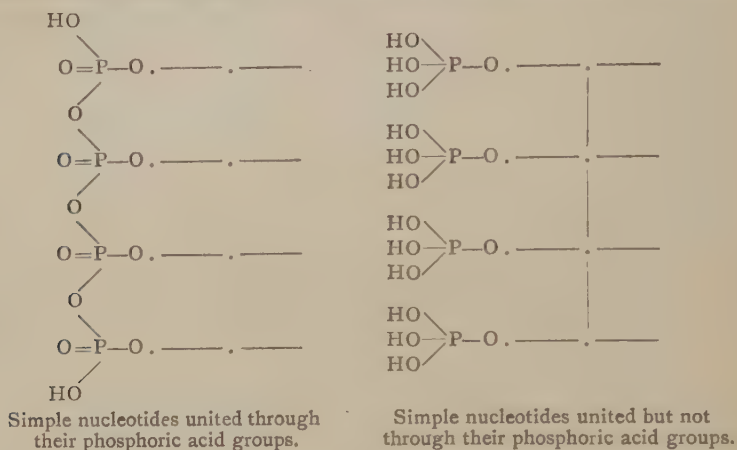
The lower curve of Fig. 2 was constructed from experimental data obtained with yeast nucleic acid, and it practically coincides with the upper curve.

This coincidence points to a very definite conclusion. If it be granted that the rates are expressions of phosphoric acid linkages, then the phosphoric acid linkages of yeast nucleic acid must coincide with the phosphoric acid linkages of its four component nucleotides:—



If yeast nucleic acid is a chemical combination of the four nucleotides, then in this union the phosphoric acid groups of the nucleotides must not be disturbed. No additional phosphoric acid linkages can be introduced. The four nucleotides possess together eight replaceable

hydrogen atoms; so also, yeast nucleic acid must contain eight replaceable hydrogen atoms. Therefore the nucleotide linkages of yeast nucleic acid cannot be through the phosphoric acid groups: the linkages cannot involve any one of the four phosphoric acid groups.¹



II.—A comparison of the acidity of yeast nucleic acid with the acidity of its nucleotides (Jones [1920, 2]).

Pig's pancreas contains a variety of active agents (ferments) which decompose nucleic acid and further act on its decomposition products. When an aqueous extract of pancreas is boiled, all of these active agents are destroyed but one, viz. the one that decomposes yeast nucleic acid into its nucleotides.

An aqueous extract of pig's pancreas was boiled and filtered. Yeast nucleic acid was added to the clear extract, and the mixture was allowed to digest for 20 hours at 40°, when nucleic acid had disappeared and a mixture of the four nucleotides could be isolated.

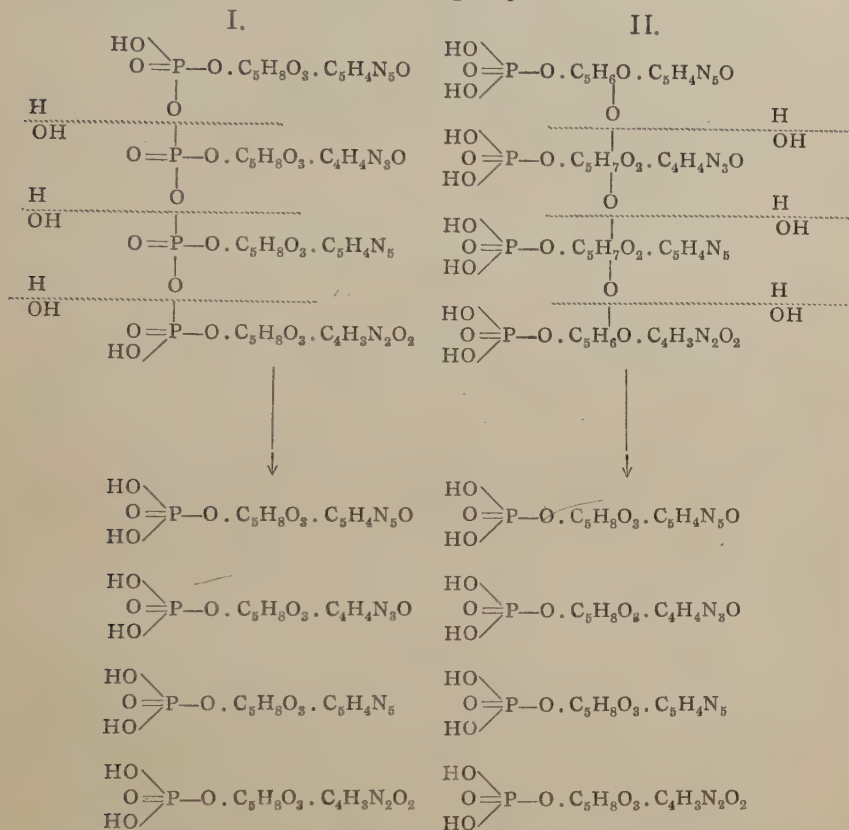
This ferment (if it is a ferment) acts rather rapidly at first, but more slowly afterwards. It is exceedingly more active at 40° than at 20°, and exhibits about the same activity whether the solution be amphoteric, faintly alkaline, or acid to litmus. It is not present in the liver nor the spleen, and does not decompose thymus nucleic acid.² In the decomposition of yeast nucleic acid by this ferment, neither phosphoric acid nor purine bases are set free, and deaminization does not occur.

But when yeast nucleic acid is converted into its nucleotides by

¹ The argument here used is essentially that which was employed for the same purpose by Jones and Read [1917, 2]. Their reasoning is perfectly sound.

² This is curious. An active agent is present in animal pancreas which is specifically adapted to plant nucleic acid. It suggests evolutionary matters.

this active agent, there is not the slightest change in the acidity of the solution. Two formulas for nucleic acid are given below which differ from one another in only one respect. In No. I., the nucleotide linkage is through the phosphoric acid groups; in No. II. the nucleotide linkage is through the carbohydrate groups.



If Formula I. correctly represents the structure of nucleic acid, the conversion of nucleic acid into its nucleotides should be attended by a marked increase in acidity¹; the increased acidity caused by the decomposition of two grams of nucleic acid should require about 8 c.c. of tenth normal sodium hydroxide for neutralization toward phenolphthalein.

On the other hand, if Formula II. correctly represents the structure of nucleic acid, the conversion of nucleic acid into its nucleotides should not be attended by any increase in acidity.

As a matter of fact, when two grams of nucleic acid are decom-

¹ There is no doubt about this. The nucleotides imitate the conduct of phosphoric acid toward alkalis and indicators. The two replaceable hydrogen atoms of adenine nucleotide imitate the two most highly ionized hydrogens of phosphoric acid (Jones [1920, 1]).

posed into nucleotides, no change in acidity can be detected with sensitive indicators. This is a crucial experiment that decides definitely against the phosphoric acid linkage (Formula I.). Until very recently every one conceded that the nucleotide linkages of nucleic acid must be through the carbohydrate groups, if they are not through the phosphoric acid groups. Levene and Jacobs [1912, 2] assumed this as a matter of course when they were dealing with thymus nucleic acid, and Feulgen [1918, 2] makes the statement in so many words. The following experiments confirm this unanimous opinion.

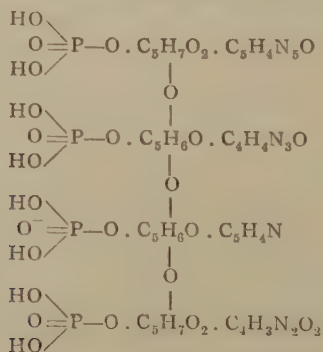
III.—A comparison of the rate at which the purines are set free from yeast nucleic acid with the rate at which the purines are set free from the individual purine nucleotides (Jones [1920, 1]).

At the time the phosphoric acid studies were made with guanine nucleotide (Jones and Read [1917, 4]) and adenine nucleotide (Jones [1920, 1]), the rates at which they set free their guanine and adenine were ascertained. It is exceedingly rapid, and the same for both purines and for yeast nucleic acid. The rapidity is such that the time required to dissolve the nucleotide in the hydrolytic agent has to be considered, and in the case of yeast nucleic acid the other products of hydrolysis make the determination of the liberated purines very difficult. But one can conclude from the data without hesitation that the purines are set free from nucleic acid, and from the individual purine nucleotides with the same rapidity.

Again, the exceeding slowness with which the pyrimidines are split by hydrolysis from yeast nucleic acid makes quantitative work impossible. But the same slowness characterizes the pyrimidine nucleotides.

Therefore the argument which was used above to show that the nucleotide linkages of yeast nucleic acid do not involve the phosphoric acid groups, may now be used to show that the nucleotide linkages do not involve the purine groups, nor probably the pyrimidine groups.

This leaves the carbohydrate groups and indicates the formula :—



Triticonucleic Acid.

There was a time in the history of the subject when this substance stood as the most completely and accurately examined of all nucleic acids. Osborne and Harris [1902], who were the first to isolate triticonucleic acid from the wheat embryo, found that its fundamental hydrolytic products are the same as those of yeast nucleic acid. Guanine and adenine were obtained *in equivalent quantities*.

The statement is often made and always granted that nucleic acids give equivalent quantities of the two purine bases. This is undoubtedly true, but it would be difficult to find published analytical data that support the statement.

Osborne and Harris also found pentose and uracil¹ among its hydrolytic products, noted the close resemblance of triticonucleic acid to yeast nucleic acid and cautiously suggested the identity of the two substances. Their opinion has been fully justified by recent investigations.

By hydrolysis with ammonia at 145°, triticonucleic acid produces the nucleosides of yeast nucleic acid.

Guanosine.	Levene and La Forge [1910].
Adanosine.	" " " " "
Cytidine.	" " " " "
Uridine.	Read and Tottingham [1917].

By hydrolysis with ammonia at 110° it produces the nucleotides of yeast nucleic acid (Read and Tottingham). Its pentose is d-ribose (Levene and La Forge), the characteristic carbohydrate of yeast nucleic acid: and its phosphorus is distributed as in yeast nucleic acid.

There is little room to doubt that the two plant nucleic acids are identical substances.

¹ In the mother liquors from which Osborne and Harris had obtained uracil Wheeler and Johnson [1903, 2] afterwards found cytosine.

CHAPTER IV.

ANIMAL NUCLEIC ACIDS.

Thymus Nucleic Acid.

KOSSEL and Neumann [1894, 2] found that two nucleic acids could be prepared from the thymus gland, distinguished from one another by the fact that one of them (α -thymus nucleic acid) forms a sodium salt whose warm aqueous solution gelatinizes upon cooling, while the sodium salt of the other (β -thymus nucleic acid) does not. Neumann afterwards described a method of preparing the two acids [1899], and Araki [1903, 1] supposed that one of the acids could be converted into the other by the action of a ferment present in the thymus gland. But it has been found that the conversion of the α -acid into the β -acid is attended by a loss of a large proportion of its purine groups (Kostytschew [1903]), or in other words, that β -thymus nucleic acid is an undefined decomposition product of the α -acid. The same is true of Neumann's [1898] nucleo-thymic acid which represents simply an indefinite stage in the decomposition of thymus nucleic acid by removal of its purine groups, a decomposition which ends in the formation of thymic acid, another poorly defined substance which probably contains no purine groups at all (Kossel and Neumann [1896-7], Steudel and Brigl [1911], Feulgen [1918]).

Neumann may have been dealing with two different sodium salts of thymus nucleic acid, one of which gelatinizes and the other does not. The neutral sodium salts of all nucleic acids are gelatinous and can be alternately liquefied and gelatinized by the alternate addition of acid and alkali to their aqueous solutions (Jones [1908]).

The writings of Bang have unfortunately tended to complicate the chemistry of thymus nucleic acid. His remarkable finding of glycerine [1901] among the hydrolytic products of guanylic acid (see Chapter III.) and his subsequent contributions on the nucleoproteins [1904]: [1905]: [1906-7] culminated in the assumption that the thymus gland contains two nucleic acids, one of which produces both amino-purines, and the other only adenine. This conclusion was based simply on the finding of approximately twice as much adenine as guanine among his hydrolytic products, which was an evident experimental error, since all other experimenters who have made careful estimations of the purine bases obtainable from thymus

nucleic acid have found them present in approximately equivalent quantities (Steudel [1906, 1]: [1906, 2]: [1907], Jones and Austrian [1907, 1], Jones [1908]).

So far as any evidence to the contrary is concerned, the thymus gland contains but one nucleic acid.

The Structure of Thymus Nucleic Acid.

One of the very few attempts to obtain decomposition products of thymus nucleic acid other than those of hydrolysis was the examination of the products formed by the oxidizing action of calcium permanganate (Kutscher and Seeman [1903, 1]: [1903, 2], Kutscher and Schenck [1905]). Adenine, urea, biuret, oxalic acid, formic acid, acetic acid and butyric acid were found. From their failure to find uric acid Kutscher curiously concluded that uric acid is not formed in the animal body from nucleic acid but probably in some synthetical way. But he should not have expected to find uric acid under the conditions of his experiment (Burian [1904-5, 1]), for by the action of permanganate, uric acid is decomposed into oxalic acid and urea (Claus [1874]), two products which Kutscher obtained. The results of this oxidation experiment thus throw no light upon the structure of nucleic acid.

The same is true of Burian's effort [1904]: [1907, 1] to show that the purine rings are joined in nucleic acid at position seven. Though Burian's work is only a modest contribution to this most complex subject, his results have been called seriously into question by subsequent writers (Steudel [1906, 1], H. Fischer [1909]).

As stated in the last section various products of the partial hydrolysis of thymus nucleic acid have been described, but they are so indefinite as to be of little use in considerations of structure.

More recently Steudel has published a number of papers which he thinks contain experimental data sufficient to establish the constitution of thymus nucleic acid, and he does not hesitate to write a structural formula for the substance. But how he could have been led to this last procedure from the facts in his possession is a little difficult to see. His work is undoubtedly a very thorough overhauling of thymus nucleic acid, but he adds practically nothing to the facts that were already conceded and which had not been considered in any degree adequate for the purpose to which Steudel puts them (Steudel [1904]: [1904-5]: [1905]: [1906, 1]: [1906, 2]: [1907]: [1908]: [1912]).

The results of Levene and his collaborators deserve careful

consideration. They proceed upon the assumption that thymus nucleic acid is constituted after the general pattern of the constitution of yeast nucleic acid. Analogies are sometimes misleading but this one is supported by a mass of evidence. The two nucleic acids possess four identical fundamental groups (phosphoric acid, guanine, adenine, cytosine). The fifth is a carbohydrate group and the sixth is a pyrimidine group in each nucleic acid. This similarity of fundamental groups is very striking.

Again, the two nucleic acids conduct themselves in a very similar manner when brought in contact with various ferments at the body temperature. Levene and Medigreceanu [1911, 2] observed the optical changes produced in solutions of the two nucleic acids and noted that while animal nucleic acid is more resistant to enzymatic decomposition, the changes so produced in its optical properties are parallel to the corresponding changes of plant nucleic acid.

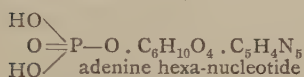
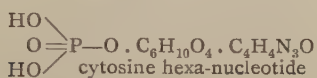
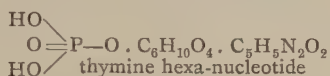
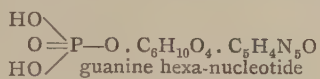
So also in all ferment studies the two nucleic acids are interchangeable. It will be pointed out that an aqueous extract of dog's liver can deaminize combined adenine but not free adenine (Amberg and Jones [1911, 2]); a quality of the extract that may be demonstrated equally well with either of the two nucleic acids. It will also be shown that an aqueous extract of pig's pancreas can deaminize combined adenine but not combined guanine. This also may be demonstrated with either nucleic acid. Such instances of the similar or identical behaviour of the two substances could be greatly multiplied.

On the other hand the two nucleic acids show occasional differences in their conduct to chemical reagents. It was seen that when subjected to hydrolysis with ammonia, yeast nucleic acid decomposes smoothly, forming in turn nucleotides and nucleosides. But thymus nucleic acid is not altered by heating with ammonia unless the temperature is raised so high as to produce deep-seated decomposition.

A similar difference between the two nucleic acids is to be observed when they are submitted to hydrolysis with boiling dilute mineral acid. Yeast nucleic acid promptly sets the purines free and the guanine may be precipitated quantitatively by the addition of ammonia. But under the same conditions, thymus nucleic acid produces substances that form an emulsion with ammonia which is very difficult to deal with. This is why the identity of the purine bases obtained from animal nucleic acids is so frequently mistaken.

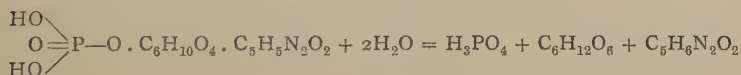
But we should not assume that two *similarly* constituted chemical compounds will give decomposition products with identical physical properties.

Taking all things into consideration a resemblance between the two nucleic acids is found at every point where it could reasonably be expected, and if the constitutional similarity between the substances be granted, then the results of Levene become both clear and important: for we must regard thymus nucleic acid as a tetra-nucleotide composed of groups of four mono-nucleotides. It is necessary to call them *hexa*-nucleotides in order to distinguish them from the corresponding *penta*-nucleotides of yeast nucleic acid:—



By partial hydrolysis of thymus nucleic acid Levene and Mandel [1908, 3] obtained a substance of the composition $\text{C}_{11}\text{H}_{17}\text{N}_2\text{PO}_{10}$, which was decomposed by severe hydrolysis into phosphoric acid, hexose (i.e. *lævulinic acid*) and thymine.

The compound is evidently thymine nucleotide,



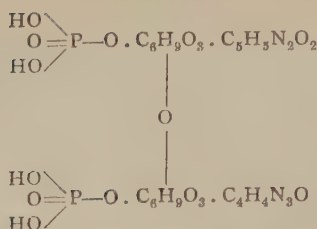
Levene and Jacobs [1912, 1] subsequently obtained from animal nucleic acid by ferment action a substance having the formula, $\text{C}_{11}\text{H}_{15}\text{N}_5\text{O}_6$, which by hydrolysis forms guanine and hexose:—

The compound can be no other than guanine nucleoside,

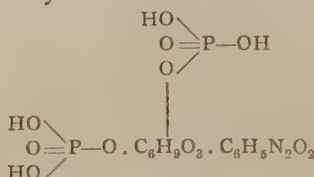


The nucleotide linkages of thymus nucleic acid are inferred from three substances which Levene and Jacobs [1912, 2] prepared from the nucleic acid of fish sperm by partial hydrolysis with dilute sulphuric acid.

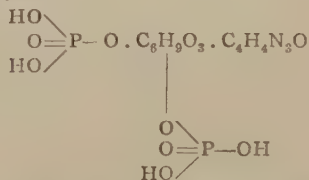
1. *A Di-nucleotide of Thymine and Cytosine*.—Levene and Jacobs discuss this substance very briefly without even stating how it is prepared. They do note, however, that the substance is a tetrabasic acid and for this reason its nucleotide linkage cannot involve the phosphoric acid groups. They therefore conclude that it must be between the carbohydrate groups and assign the formula



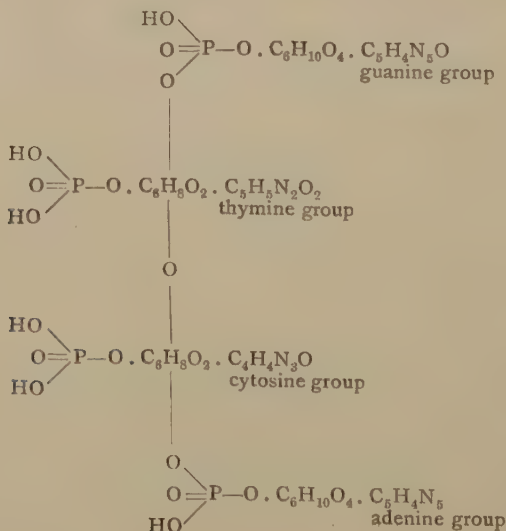
2. *Hexa-thymidine-di-phosphoric acid* which forms a crystalline brucine salt and produces phosphoric acid, lævulinic acid and thymine by severe acid hydrolysis:—



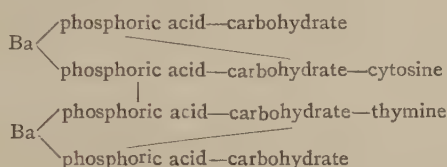
3. *Hexa-cytidine-di-phosphoric acid* which forms a crystalline brucine salt and produces phosphoric acid, lævulinic acid and cytosine by severe acid hydrolysis:—



Relying principally upon the structures of these three compounds, Levene and Jacobs assign to thymus nucleic acid the following structural formula:—



Feulgen [1918, 2] does not concede that the central nucleotide linkage of thymus nucleic acid is between the carbohydrate groups as expressed in this formula, but claims that the two pyrimidine nucleotides are united through their phosphoric acid groups. His argument is rather indirect. He believes he has found a satisfactory method of removing the purine groups of thymus nucleic acid without disturbing the rest of the molecule, and looks upon the substance so formed (thymic acid) as differing from thymus nucleic acid only at the one point. He ascribes to its barium salt the formula—



If thymic acid (Feulgen [1918, 1]) is a tetrabasic acid as shown by the composition of its barium salt, so also thymus nucleic acid must be a tetrabasic acid, and its central nucleotide linkage can only be through its phosphoric acid groups.

But what is thymic acid? It was prepared from thymus nucleic acid by a mild hydrolysis of the substance with dilute sulphuric acid and it should consist to some extent of a mixture of the preparations of Levene and Jacobs described above.

PART II.

THE PHYSIOLOGICAL CONDUCT OF NUCLEIC ACIDS.

The Formation of Nucleic Acid in the Body.

THE discovery of nucleic acid in the spermatozoa heads of the Rhine salmon carried with it the proof that nucleic acid is not formed in the body from purine precursors. During the entire period consumed in ascending the river these fish never partake of food; at least the alimentary tract is never found to contain food detritus and the digestive fluids are practically inactive. The great increase, therefore, in the nuclein-rich generative organs, going hand in hand with a decrease in muscle tissue, must mean that nucleic acid is formed directly or indirectly from muscle protein (Miescher [1874, I]).

More direct proofs became possible when it was found that purine bases are formed from nucleic acid and may serve for its detection. Tichomiroff [1885], in Kossel's laboratory, availed himself of the method thus suggested and found that the eggs of insects take on purine material as development proceeds; and somewhat later Kossel [1886] made a closer examination of the hen's egg in this respect [1886]. Before incubation the presence of combined purines could not be demonstrated in the entire yolk,¹ but after incubation had proceeded for fifteen days, purine material had made its appearance. Kossel's work was so satisfactory that the question did not again arise for eleven years, when Burian and Schur [1897] observed that no experiments had hitherto been made with mammals and proceeded to supply the omission. Of two newly-born rabbits the one was immediately killed while the other was allowed to grow with milk as its only source of nitrogenous food. As milk is so nearly free from purine derivatives as to be out of consideration as a source of purine material for the cell nuclei,² any increase of combined purines in the organs of the animal during growth must be attributed to the non-purine nitrogen

¹ Plimmer and Scott [1909] found more recently even in fresh eggs substances which contain phosphorus and resemble nucleic acid.

² This is not strictly the case, for Voegtlin and Sherwin [1918] have found in cow's milk more than traces of both guanine and adenine.

of the milk. The organs of the grown rabbit were then compared with those of the young one. The marked difference in the combined purine bases of the two is seen in the following data :—

Weight of Rabbit at Birth.	Weight of Rabbit at Time of Analysis.	Purine Nitrogen.
I. 46.7 g. II. 47.0 g.	46.7 g. 204.0 g.	0.0274 0.1215

Roughly the combined purine bases increase in proportion to the increase in body weight on a purine free diet.

The formation of nucleic acid from protein (or rather the growth of animals which necessitates nucleic acid formation) was shown by Osborne and Mendel [1912] in connexion with their exact studies of protein metabolism. The growth of normally fed rats was compared with the growth of the animals when supplied with food deficient in certain protein groups (plant proteins poor in lysine and tryptophan). The results showed strikingly that certain protein groups must be present in the food if normal increase in body weight is to follow, but that the growing body makes no such demand for purine material.

Benedict [1916] has furnished the proof that an adult mammal can synthesize a purine ring. He fed a Dalmatian dog for a year on purine free food and in that time isolated 100 grams of uric acid from the urine. Not a tenth of this could have come from the purines of the food.

But as to the identity of the purine precursor, scarcely a conjecture could be made until Ackroyd and Hopkins [1916] found that the allantoin of rats' urine is markedly diminished when the animals are placed on a diet containing neither a histidine nor an arginine group. This work appears very conclusive if we assume that allantoin can only come from uric acid.

The Physiological Decomposition of Nucleic Acid.

Consideration of protein metabolism in its relation to the chemical behaviour of proteins outside the body would suggest that in dealing with the catabolism of nucleic acid we would have to do with the laboratory decomposition products of the substance; and a close examination of the matter remarkably confirms this supposition. One is therefore concerned with the disruption of nucleic acid in the body and the fate of its six components: 1, phosphoric acid; 2, carbohydrate; 3, guanine; 4, adenine; 5, cytosine; 6, thymine. Until very recently it

was supposed that nucleic acid undergoes complete disruption by a physiological agent called "nuclease" with the contemporaneous formation of all six of its hydrolytic products. The complete decomposition of nucleic acid by tissue extracts, or the presence of nuclease in these extracts, was therefore considered proven when the liberation of any one of the six substances could be shown. Phosphoric acid was naturally selected as one of the easiest to detect, but the compound is so widely distributed in the organism that its presence can mean a decomposition of nucleic acid only under certain carefully observed conditions. On the other hand, the purine bases can easily be detected by the gelatinous precipitate which they produce with an ammoniacal solution of silver nitrate; and as they do not exist free in animal glands their presence can be regarded as an indication that nucleic acid has been decomposed.

Purine bases have been observed in animal tissues from early times but their presence could not be rationally interpreted prior to the discovery of nucleic acid; so that a great deal of the older work, even including that of Schützenberger [1874], was not intelligible until later. It is difficult at the present day to assign proper credit to the earlier investigators or to state just when the discovery of nuclease occurred. The idea seems to have been a gradual growth.

Kossel was the first to show the liberation of phosphoric acid from nucleic acid by physiological agents, but while perhaps proving the point, his data were not striking.

Salomon [1881] examined the auto-digestion products of various gland extracts and found a considerable increase of what he supposed to be hypoxanthine as digestion proceeded at the room-temperature. This was specially noticeable with extracts of pancreas and liver. Salomon was able to show that the combined or "masked" purine of the gland extract became liberated by the action of a physiological agent just as when the gland extract was boiled with mineral acid. He was familiar with Kossel's work on nuclein and showed his rather clear understanding of the matter in the following language: "Man ist daher genötigt, in der Leber eine Substanz anzunehmen, welche durch die Aktion eines ihr angehörigen, über den moment des Todes hinauswirksam Fermentes, wie auch bei der Spaltung durch Säuren Xanthinkörper angibt . . . und ich halte es ebenfalls für wahrscheinlich, dass die hypoxanthinbildende Substanz das Nuclein ist".

Lehmann [1885] studied the difference between the autolytic and hydrolytic purine bases of yeast. His results did show differences between the two processes, sufficient to indicate an autolytic action

of yeast on its own nucleic acid, but at the time of his work adenine was unknown, and methods for separating the purine bases from one another were so crude as to confuse his work past modern interpretation.

In the year 1888 Salkowski introduced chloroform as a preservative and examined the auto-digestion products of various gland extracts and of yeast under antiseptic conditions [1889]: [1890]. While his studies were broader and more accurate, they were little more than a confirmation of the previous work of Salomon: but Schweining [1894] found that nuclease is present in cell-free filtered organ extracts, and Biondi [1896] professed to prove that the ferment is different from trypsin.

In 1898 Okerblom examined what he called the xanthine bases of the suprarenal gland. He undoubtedly supposed that the substances are preformed in the gland, for he says nothing of their origin, but at the end of his contribution adds simply that more purine material can be obtained from digested glands than from fresh ones. Okerblom thought he found methyl-purines in the suprarenal gland, but this was afterwards shown to be an error by Jones [1904, 2].

Hahn and Geret [1900] found that mechanical agitation of digesting gland extracts materially assists the action of nuclease. Every one will readily grant the truth of their conclusion.

Iwanoff [1903] proved conclusively that various moulds (*Penicillium glaucum* and *Aspergillus niger*) can decompose thymus nucleic acid with liberation of both phosphoric acid and purine bases. The moulds were grown upon previously prepared nucleic acid so that the origin of the products could be definitely stated, and as no active agent was present capable of liquefying gelatin, Iwanoff concluded that he could not be dealing with trypsin and called the ferment "nuclease". He was the first writer to employ the term and is commonly credited with the discovery of the ferment. It is very certain that at the time of his publication no vitally important contribution had been made to the subject since the work of Salomon.

Shortly afterwards Jones [1904, 1] gave a clear demonstration of nuclease in animal tissues and at the same time proved that the ferment is different from trypsin. By alternate solution of the "nucleoprotein" of the thymus in dilute alkali and precipitation with acetic acid a product was obtained which contained no trace of either free phosphoric acid or free purine base. The ferment of the gland had evidently followed the nucleoprotein, for the final product was found enzymatically active and produced both phosphoric acid and purine bases when digested at 40°. But when the nucleoprotein is dried with alcohol

and ether, its activity is destroyed and the inactive product gives neither phosphoric acid nor purine bases even when digested with active trypsin.

Schittenhelm and Schrötter's [1903]: [1903-4] work of this period on the bacterial decomposition of nucleic acid scarcely concerns us. It made no addition to the existent knowledge of nuclease except that nucleic acid was found to evolve gas under the influence of putrefactive bacteria, a finding that Oppenheimer [1904] does not concede.

Nakayama [1904] found that so far as concerns its power to liberate purine bases and phosphoric acid from nucleic acid, nuclease is not to be distinguished from erepsin which was found capable of decomposing the nucleic acids of spleen, intestine, thymus, and fish sperm. One may conclude from these results that erepsin solutions free from nuclease are not easily prepared.

About the time of Iwanoff's work a new meaning became attached to the term nuclease.

Araki [1903, 1] found that an aqueous extract of thymus can convert α - into β -thymus nucleic acid, the only point of distinction between the two substances being that the sodium salt of the one gelatinizes while that of the other does not. But Schmiedeberg [1907] observed that one is justified in assuming no more than that one of the nucleic acids is an anhydride of the other, and Jones [1908] subsequently found that nucleic acid forms at least three different sodium salts, only one of which gelatinizes, and that these three salts can easily be converted back and forth into one another.

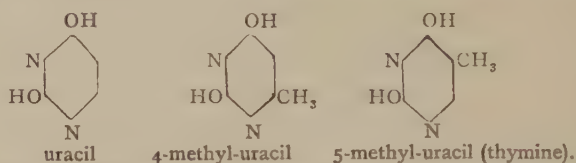
Sachs [1905], who continued the investigation of nuclease along the same line, found that as pancreas extract digests at 40° its trypsin increases while its power to liquefy gelatinous sodium nucleate decreases. Sachs concluded that trypsin destroys nuclease and that the two ferments cannot therefore be identical. This does not prove that trypsin destroys *nuclease* and the difference of the two ferments had already been established by the contributions of Biondi, Iwanoff, Jones and Nakayama. Sachs makes a number of observations about conditions which influence the activity of nuclease, but finally concedes that in the passage from α - to β -thymus nucleic acid both phosphoric acid and purine bases are removed. The published results upon this subject therefore simply mean that the decomposition products of thymus nucleic acid do not gelatinize.

This phase of the nuclease question has again been taken up by de la Blanchardière [1913], who shows, by most carefully conducted experiments, that a solution of gelatinous sodium nucleate becomes less

The subject of nuclease is a most important one and will be more fully discussed in a later section (p. 91).

The Metabolism of the Pyrimidine Derivatives.

The first experiments upon this subject were made by Steudel [1901, 2]: [1903]. He fed dogs with various pyrimidine derivatives, including a number of intermediate compounds that occur in Behrend and Roosen's synthesis of uric acid from 4-methyl-uracil. Steudel reported that 4-methyl-uracil passed the dog's organism unchanged, but that both thymine and uracil are burned in the body and cause an increase in the excretion of urea. It appeared most interesting that the position of a methyl group in a pyrimidine ring should cause such a difference in metabolic conduct:—



But unfortunately Mendel and Myers [1910] were not able to confirm Steudel's results.

Kutscher [1901-2] made the first careful examination of the pyrimidine products formed by the action of gland extracts on nucleic acids. Finely ground thymus was mixed with water and allowed to digest for a month at 40°. From 500 grm. of gland he obtained 0.6 grm. of a crystalline derivative whose analysis gave numbers intermediate between those of thymine and uracil, and Kutscher thought he had possession of a mixture of these two substances. He probably thought also that the same two pyrimidine derivatives are formed by hydrolysis of thymus nucleic acid; at least this idea was commonly held at the time.

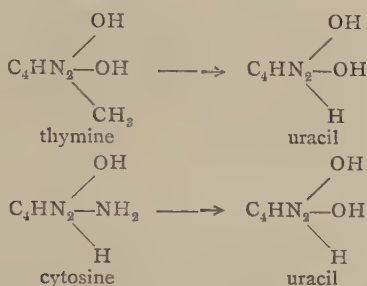
Reh [1903] had the same experience with lymph glands, but upon recrystallization of his product he finally obtained a substance that had a nitrogen percentage even greater than uracil. (Probably cytosine.)

Among the autolytic products of the pancreas, Levene [1902-3, 2] found uracil but only a questionable trace of thymine, while with liver, uracil alone was obtained. Levene was of the opinion that in the autodigestion of these glands thymine was formed first but was afterwards converted into uracil.

Upon repetition of Kutscher's work with autodigested thymus, Jones [1904, 2] obtained a pyrimidine product which formed crystals of

needle clusters and contained the amount of nitrogen required for uracil : but neither cytosine nor thymine could be found.

All of these results were formerly explained by assuming that the glands in question contain two ferments, one of which causes demethylation of thymine, and the other, deaminization of cytosine. In both cases uracil would be formed :—

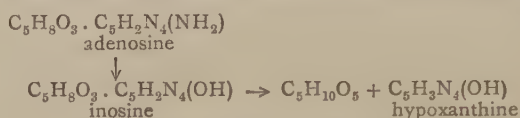


But Levene [1904] found this explanation inadequate when he recovered *free* thymine unchanged after digestion at 40° with an aqueous extract of pancreas. Sweet and Levene [1907] also recovered the substance from the urine of a dog that had been fed with it : so that the absence of thymine among the products of glandular autolysis came to be looked upon simply as an analytical loss and the presence of uracil was thought to be due only to the deaminization of cytosine.

In this connexion an unpublished experiment of Jones is of interest. A specimen of fresh spleen extract was divided into two equal parts and one portion was sterilized by boiling. To each was added 300 mg. of cytosine chloride and after digestion at 40° the products were examined for pyrimidine derivatives by the ordinary method which always involves considerable loss of material. From each experiment was obtained an amount of cytosine picrate which corresponds to about 200 mg. of cytosine chloride. Thus while the spleen extract was formerly found to produce uracil on self-digestion it cannot convert free cytosine into uracil ; and it would seem that the tissue possesses neither of the two functions formerly ascribed to it.

The metabolism experiments of Mendel and Myers [1910] show that the free pyrimidine derivatives pass the dog's organism unchanged. They record the first experiments made with cytosine which they recover from the urine after giving to dogs and rabbits by subcutaneous injection. They find the same inability of these animals to alter thymine or uracil, and so far as the free pyrimidines are concerned, one is safe in concluding that the organism does not possess an agent that can bring about demethylation or deaminization. But Mendel and Myers observe that although the free pyrimidines pass the organism

unchanged, they cannot be found in the urine after feeding nucleic acid. This suggests that the substances may undergo alteration in the body or in tissue extracts so long as they exist in combined form. In the case of purine derivatives this has been found true. Jones [1911, 2] and Amberg and Jones [1911, 2] have shown that the adenine group of nucleic acid is completely deaminized by the action of an extract of dog's liver so that when nucleic acid is digested with the tissue extract the theoretical amount of hypoxanthine is produced. The deamination of adenine nucleoside is probably what occurs, for the tissue extract cannot convert free adenine into hypoxanthine:—



But in the case of the pyrimidine compounds, if any alteration occurs it is not while the substances are in the form of nucleosides. Levene and La Forge [1913] find that uridine is not altered by ferment action and even hydro-uridine which yields easily to acid hydrolysis, is perfectly stable to ferments.

This leaves the question of pyrimidine fermentation in a most unsatisfactory condition. If the pyrimidine derivatives cannot be liberated from nucleic acids by physiological agents, how came the pyrimidines which have surely been in the possession of a number of experimenters who obtained them as autolytic products? They cannot be laboratory products. It seems that they must have been liberated from nucleic acid before the nucleosides were formed.

The Formation of Uric Acid from Nucleic Acid.

Uric acid was formerly believed to be an intermediate product of protein metabolism, but the discovery of purine bases as decomposition products of nucleic acid at once suggested that uric acid is formed from these specific groups. The characteristic chemical structure possessed in common by the nuclein bases and by uric acid could not fail to give the impression that a physiological connexion exists between them and it was not long after the discovery of the nuclein bases that attempts were made to place this connexion on an experimental basis.

The question was first taken up by Stadthagen [1887] who fed yeast nucleic acid to dogs, but for some reason which cannot be stated he found that the ingested nucleic acid did not cause an increased excretion of either uric acid or purine bases, although an

increase in the output of phosphoric acid showed that the nucleic acid had been absorbed from the digestive tract. Stadthagen also fed dogs with guanine, but again failed to find any increase in the uric acid of the urine. This work is interesting because the experimenter had evidently undertaken his work with the conviction that uric acid *is* physiologically derived from nucleic acid: but it is even more remarkable that seven years later Gumlich [1894] confirmed Stadthagen's error.

But it was not long before the metabolic formation of uric acid from nucleic acid was shown in such a way as to remove all doubt that may have been raised by Stadthagen's work. Minkowski [1886] had found that the urine of birds with extirpated liver contains ammonium lactate which evidently replaces the uric acid present under normal conditions. But although extirpation of the liver caused a great diminution in the excretion of uric acid the substance never entirely disappeared from the urine. This suggested that in birds uric acid is derived from two sources: the one, ammonium lactate which is converted into uric acid in the liver, and the other, some unknown precursor by a process in which the liver takes no part. Proceeding upon this assumption, v. Mach [1888] injected hypoxanthine subcutaneously into geese with extirpated livers and found the substance converted into uric acid which appeared in the urine. The ability of the animal organism to form uric acid from a purine precursor was thus shown for the first time, but the origin of uric acid was not traced to nucleic acid.

This remained for Horbaczewski [1889]: [1891] whose investigations are so fundamental as to require most careful examination. Spleen pulp of the calf was mixed with water and allowed to digest at 50° until putrefaction had set in. The fluid was then sterilized with a solution of lead acetate, and after the addition of arterial blood was kept at 50° while a slow current of air was passed through the digesting mixture. At the end of the operation the fluid was found to contain uric acid, while similar experiments without the passage of air resulted in the formation of xanthine and hypoxanthine instead of uric acid.

Horbaczewski's work is so acceptable as to be above adverse criticism, yet the following points should be noted:—

1. He believed the blood to play an important part in the formation of uric acid, but in reality his use of arterial blood was entirely superfluous.

2. He thought putrefaction a necessary factor and was careful to see that putrefaction had occurred.

3. He had no idea that he was dealing with ferments present in the spleen pulp itself, for experiments were even made with "nuclein" obtained by gastric digestion and dried with alcohol and ether. The use of lead acetate for *sterilization* also shows that the possible destruction of ferments was not considered.

4. He believed that the "potential" formation of uric acid occurred *before* the purine groups were set free from the nucleic acid, and specifically states that the uric acid is not produced by the oxidation of *free* xanthine or hypoxanthine.

5. He obtained uric acid with tissues that are now known to be free from the oxidizing ferment that is indispensable to its formation. Calf's thymus, which surely does not contain this ferment, was found to yield more uric acid than any of the large number of organs examined, and it was even stated that human organs do not differ from calves' organs in their ability to form uric acid.

It is possible that Horbaczewski was imposed upon, for his principal experiment (the one with ox spleen) was afterwards *exactly* repeated by Spitzer [1899] and confirmed.

Horbaczewski also showed the formation of uric acid from nucleic acid in the organisms of the rabbit and man, but he believed the uric acid to be formed from dead leucocytes and that the ingested nuclein contributed to the formation of uric acid only so far as it induced leucocytosis. He had observed an increased excretion of uric acid following the alimentary leucocytosis which occurs when feeding is resumed after starvation; in fact his experiments were undertaken for the reason that patients with leucæmia whose blood showed a high leucocyte count, had been found to excrete an unusually large amount of uric acid.

Horbaczewski's leucocytosis theory has been the subject of wide comment and has been alternately supported and contradicted by subsequent observers, but has finally been almost entirely abandoned (see Burian [1904-5, 1]). Nevertheless his experimental findings have been amply confirmed. Increased excretion of uric acid after the ingestion of nucleic acid or food rich in nucleic acid has been observed by Kühnan [1895], Richter [1895], Weintraud [1895], Umber [1896], Hess and Schmoll [1896], Cohn [1898], Weiss [1899], Loewi [1901], Mendel and Brown [1900], and many others, while Luethje [1896] states that he could find no limit to the ability of the dog's organism to form uric acid from fed thymus. The objections raised by Hopkins and Hope [1898-9] have been considered by Jerome [1899-1900]. They may have been pertinent at the time

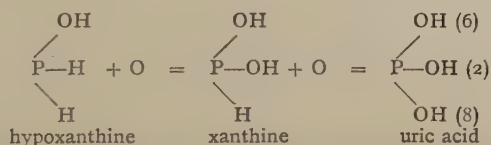
they were published but have lost force through subsequent discovery.

While the formation of uric acid from the combined purine bases as they exist in nucleic acid has been conclusively demonstrated all of the earlier attempts to bring about an increased excretion of uric acid by ingestion of free purine bases had failed. Kerner [1857], Nencki and Sieber [1883], Baginski [1883-4], Krüger and Salomon [1895-6, p. 184], Burian and Schur [1900]. It is true that these experiments were all made with guanine or xanthine which are absorbed from the digestive tract with great difficulty and may therefore have been lost (Hall [1903-4]). But even where special care has been taken to prevent loss in this way, as by subcutaneous injection (Schittenhelm and Bendix [1904-5]), the results have been far from satisfactory.

It therefore becomes necessary to examine closely the process by which uric acid is formed from nucleic acid. It will be found that several factors are concerned in the transformation, and it is convenient to discuss them in inverse order to the one in which they are exerted in the tissues ; beginning with uric acid and ending with nucleic acid.

The Formation of Uric Acid from the Oxy-purines.

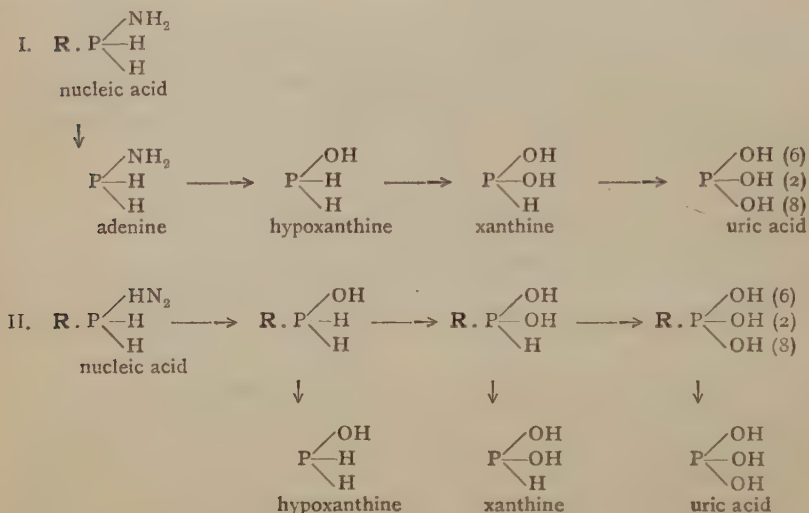
The conversion of the oxy-purines into uric acid is a simple question of oxidation :—



The presence of a ferment in the tissues which can effect this oxidation may be inferred from a number of considerations, but it can be proven in only one way. A known amount of the oxy-purine is added to an aqueous extract of the tissue in question and the mixture is allowed to digest at 40° in the presence of a sufficient supply of air. The oxy-purine must then have disappeared and in its place must be found a reasonable equivalent of uric acid. Horbaczewski [1889]: [1891] observed that uric acid can be formed from nucleic acid, but he did not believe that the process involved the intermediate formation of the oxy-purines, and expressly stated that as the free oxy-purines cannot be oxidized to uric acid the path from nucleic acid to uric acid does not lead through xanthine or hypoxanthine.

Two of the paths by which uric acid may be formed theoretically from nucleic acid are represented in the following diagrams. The

first shows the production of uric acid by three successive chemical decompositions occurring in the order—hydrolysis—deaminization—oxidation, and *involves the passage of the oxy-purines into uric acid*. The second represents Horbaczewski's idea. The chemical changes occur in the order deaminization—oxidation—hydrolysis. The loss of amino-groups was supposed to be brought about by putrefaction which, with the oxidation following, occurred while the purine groups were still in nuclein combination. This transformation *does not* involve the oxidation of the oxy-purines to uric acid:—



Horbaczewski is entitled to the greatest credit for showing the origin of uric acid from nucleic acid, but he proved nothing and knew nothing of the agents concerned nor of the intermediate products.

v. Mach [1888] was the first to show the physiological conversion of oxy-purine into uric acid, when he found that birds with extirpated livers excreted uric acid after subcutaneous injection of hypoxanthine. Minkowski [1898] afterwards went more deeply into the subject. At this time it had been uniformly found that feeding of material rich in nucleic acid is followed by an increased excretion of uric acid but that the same result cannot be brought about with free purines. Minkowski made his first experiments on dogs with adenine, and was able to confirm Kossel's [1888, 1] previous observation that the amino-purine is to some extent excreted unchanged; but severe alimentary disturbances were created which made it impossible to obtain results of any value. With hypoxanthine, however, the case was very different. The substance did not prove to be at all toxic and gave rise to a marked increase in the excretion of uric acid. Having demonstrated

that the substance is not poisonous to dogs, Minkowski fed hypoxanthine to men and here also found an increase in the output of uric acid. His results were so decisive that the matter never again came into discussion, and the free oxy-purines were permanently removed from any exceptional place in the nuclein metabolism.

But there remains the apparent anomaly which is presented by the free amino-purines. These two substances are characterized by the difficulty and incompleteness with which they form uric acid in metabolism; while the same substances in nuclein combination undergo the necessary changes with great ease. Mendel, Underhill and White [1903] have even found that the amino-purine groups of nucleic acid are deaminized and oxidized when the substance is given *per rectum*.

It is true that the conclusions of Krüger and Schmidt [1901-2] would do away with any difference in the fate of free and combined amino-purines in the human organism, but their determinations are indirect, incomplete, and quantitatively poor, while adenine was found partly excreted unchanged. Moreover, the most recent experiments show that extracts of human organs do not in any way alter free adenine.

Following the work of Minkowski, investigations were carried on with aqueous extracts of animal glands. Wiener [1899] found hypoxanthine converted to some extent into uric acid when digested at 40° with a paste of ox liver. His work was concerned principally with a different subject and will be discussed later. Contemporaneously the very thorough and decisive work of Spitzer [1899] appeared. He proved in a most satisfactory way that both xanthine and hypoxanthine are oxidized to uric acid by aqueous extracts of ox liver and ox spleen when a current of air is passed through the digesting mixture. In one experiment he obtained an amount of uric acid which corresponds to 90 per cent. of the oxy-purine employed. It was also shown that the amino-purines are to some extent similarly converted into uric acid, and while Spitzer recognized a considerable difference in this respect between the amino-purines and the oxy-purines, he concluded without hesitation that all four of the three purines are capable of forming uric acid by the action of physiological agents present in ox liver and ox spleen. Spitzer also made the important discovery that the "oxidase" is confined to certain organs, for the conversion of the oxy-purines into uric acid could not be effected with extracts of other organs of this animal species. The thymus which Horbaczewski had found specially able to form uric acid was shown by Spitzer to be entirely lacking in any ferment capable of oxidizing the free oxy-purines.

Spitzer's work has been much broadened by subsequent investigators. Burian [1904-5, 2] made a careful study of the conditions under which the oxidising ferment exerts its activity and applied to it the name xanthine-oxidase. The ferment is not widely distributed but is confined to certain organs. Its localization in a given organ is markedly different for different animal species and depends to a considerable extent on the age of the animal. This variable occurrence of xanthine-oxidase may be seen in the following table:—

Organ Extracts which contain Xanthine-oxidase.	Organ Extracts which do not contain Xanthine-oxidase.
Human liver Ox liver Ox spleen Adult pig's liver No organ of the rat	Other human organs Ox thymus (calf's) Pig's spleen Embryo pig's liver All organs of the rat

The Formation of the Oxy-purines from the Amino-purines.

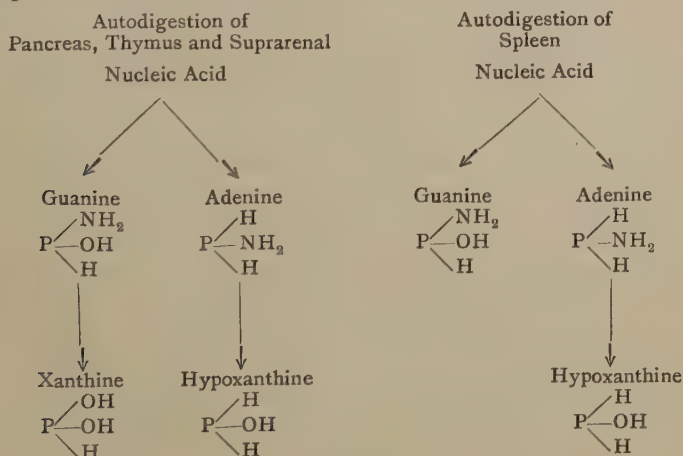
The conversion of amino-purines into oxy-purines by the action of putrefactive bacteria was early shown by Schindler [1889]. Horbaczewski [1889]: [1891] moreover found that the oxy-purines are formed from spleen pulp instead of uric acid if the passage of air be omitted; but he was most decided in his opinion that the deamination was due to putrefactive agencies which were exerted upon the nucleic acid before the purine groups were liberated (see page 65). Spitzer [1899] found that while aqueous extracts of ox liver and ox spleen can convert the amino-purines into uric acid, extracts of other glands cannot. But he did not attempt to find what did occur in the latter cases and there is nothing in his article to indicate that the amino-purines were altered in any way. It remained therefore to prove that animal tissues are provided with independent deaminases which, in the absence of xanthine-oxidase, can convert the two amino-purines into the corresponding oxy-purines.

The proof begins with the work of Okerblom [1898] who believed he found methyl-xanthines among the autolytic products of the suprarenal gland. Jones and Whipple [1902] therefore made an examination of the nucleic acid of this tissue, supposing that the gland contained a peculiar nucleic acid whose metabolism might account for the methyl-purines which Krüger and Salomon [1898-9, 1] had found in the urine; and in order to avoid any loss of methyl-purines which might occur in the preparation of the nucleic acid, the work was done with the so-called "nucleoprotein". The substance was found different in no respect from Hammarsten's [1894] α -nucleoprotein of

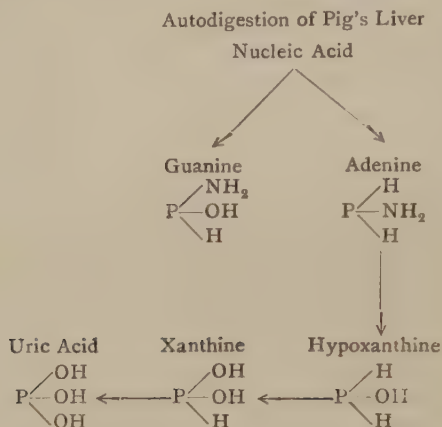
the pancreas and gave by hydrolysis the usual two purine bases, guanine and adenine. Okerblom's work was then repeated by Jones [1904, 2] and although methyl-purines were not found, xanthine and hypoxanthine were obtained where guanine and adenine might have been expected. During the digestion air was not passed lest putrefaction occur, but ample opportunity was given for the formation of uric acid by frequent agitation and venting of the vessel in which the digestion was going on. Wiener's work [1899] had shown this to be sufficient for the provision of the oxygen required for the oxidation of the oxy-purines. Yet uric acid was not found among the products. Experiments were then made by Jones [1904, 1] with pancreas and with thymus which Spitzer had found free from xanthine oxidase. Here again the products of self-digestion were found to be oxy-purines. Thus by acid hydrolysis of the nucleic acids of suprarenal, thymus and pancreas the amino-purines guanine and adenine are obtained, but in the self-digestion of the glands the corresponding oxy-purines are formed.

With spleen a curious result was obtained (Jones [1904, 2]). From the results obtained in the three cases described one might reasonably have expected the autolytic bases to be the oxy-purines; but this was not the case. One oxy-purine (hypoxanthine) and one amino-purine (guanine) were found but no trace of the complementary substances adenine and xanthine.

These results may be taken to indicate that in auto-digestion the amino-purines are first set free from the nucleic acid of the gland and are afterwards deaminized by ferments, guanase and adenase, present in the suprarenal, thymus and pancreas. If this be the case only one of the two ferments can be present in the spleen, for only one of the amino-purines is deaminized, and the two deaminases must therefore be independent of one another:—

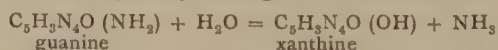


By similar reasoning, one must conclude that the liver contains adenase and xanthine-oxidase for the end products of the auto-digestion of this gland are guanine, xanthine and uric acid (Jones and Winternitz [1905], Jones and Austrian [1906]) :—

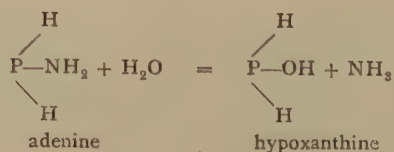


These, however, are only theoretical considerations. They are of considerable weight to be sure, and even if unsupported by other evidence might serve for the tentative assumption of two independent ferments "guanase" and "adenase". But it is unsafe to assume the existence of ferments by inference. A ferment can be proven in only one way. The substance to be decomposed must be present and disappear, while the substance supposed to be produced must be found in reasonable quantity.

Proceeding upon the assumptions stated, Jones and Partridge [1904] submitted *free* guanine to digestion with an extract of pancreas. The substance was completely changed to xanthine :—



and it seemed rather certain that those purine ferments would be found in all the glands under discussion which had been indicated by the products of self-digestion. This proved to be the case. To an extract of pig's spleen free adenine was added and the material digested at 40°. At the end of the digestion the adenine had disappeared and in its place hypoxanthine was found (Jones and Winternitz [1905]) :—



On the other hand, pig's spleen extract was similarly digested with guanine, but at the end of the digestion the substance was recovered unaltered while no xanthine could be found (Jones and Austrian [1906]). Owing to a discussion that followed, these two experiments were repeated under a variety of conditions but the results were always the same: adenine was changed to hypoxanthine, but guanine was not altered (Jones and Austrian [1906]). Finally the two amino-purines were both placed in the same specimen of pig's spleen extract and thus digested under precisely the same conditions. The adenine was deaminized, but the guanine was not (Jones [1905]). There is no escape from the conclusion that one of the two deaminases is not present in pig's spleen, and therefore two independent deaminases, guanase and adenase, are present in thymus, pancreas and suprarenal.

It should be particularly noticed that so far as pig's spleen is concerned it makes no difference whether the amino-purines are free or combined, the results are precisely the same. In self-digestion where one is dealing with the nucleic acid of the gland, that is with the amino-purines in organic combination, one obtains guanine (amino-purine) and hypoxanthine (oxy-purine), and the same substances are obtained when one starts with the two free amino-purines. While the consequences of this autodigestion experiment were acknowledged, the fundamental experiment itself was denied by Schittenhelm [1905, 2]. To concede the superstructure and deny the foundation seems a little illogical.¹

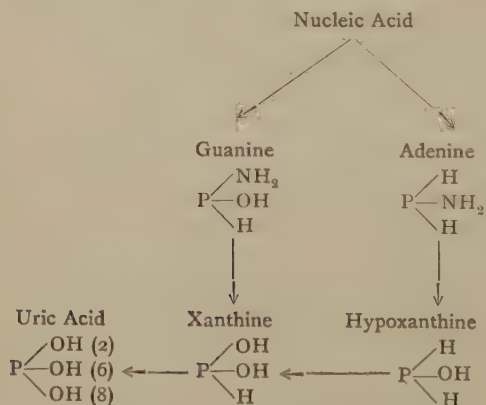
It has been claimed that guanase and adenase are but one ferment and that the peculiarity shown by pig's spleen is to be explained by assuming that adenine is more sensitive than guanine to its action. This assumption is utterly gratuitous but is nevertheless shown incorrect by the conduct of the purine ferments of dog's liver. An extract of this tissue converts guanine into xanthine with great ease but is without action on free adenine, just the reverse of the conduct of pig's spleen. Dog's liver contains guanase but not adenase: pig's spleen contains adenase but not guanase: neither tissue contains xanthine-oxidase. The three ferments are therefore independent of one another.

It is unfortunate that in the publication of the work with pig's spleen Jones failed to name the animal species. His results therefore appeared to be in direct contradiction to the previous results of

¹ Note the discrepancy between the experiments and the conclusions in a very recent article on this subject by Schittenhelm and Wiener [1912].

Horbaczewski and of Spitzer who had experimented with ox spleen. For some time the matter was puzzling, but Jones [1905] finally showed that the apparent discrepancy was really due to a difference in animal species. In the meantime Schittenhelm [1904] who had repeated and extended Spitzer's work naturally found that *ox spleen* could deaminize both amino-purines, and seeing therefore no reason for assuming the existence of two deaminases, he insisted that guanase and adenase are identical [1904-5]: [1905, 1]. Proceeding upon the assumption he undertook a somewhat reckless contradiction of the experimental finding of Jones and his co-workers (Schittenhelm and Schmid [1907, 2]: [1906, 7]), but with the assistance of the later contributions of Mendel and Mitchell [1907], Wells and Corper [1909] and others, Schittenhelm [1909]: [1910] modified his previous assumptions to a considerable extent.

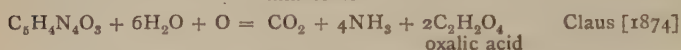
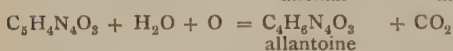
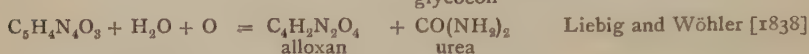
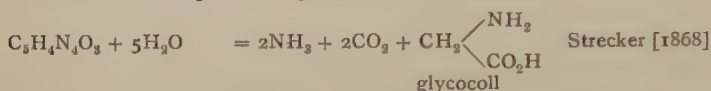
Guanase, adenase and xanthine-oxidase have been frequently the subjects of more recent investigation, and their localization in the tissues has been found to vary greatly with animal species, gland and age, and writers have been unable to formulate their experimental results on any other hypothesis than that the two deaminases are independent of xanthine-oxidase and independent of one another. It is therefore necessary to assume that the physiological formation of uric acid from nucleic acid involves at least four agents. The first sets free the two amino-purines which, by the action of guanase and adenase, are converted into the corresponding oxy-purines. These are finally oxidized to uric acid by xanthine-oxidase:—



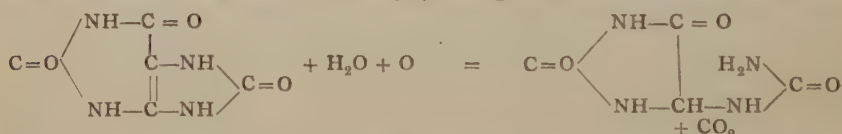
The Synthetical Formation of Uric Acid.

It has been stated that the combined results of Minkowski [1886], and v. Mach [1888] prove beyond doubt that birds produce uric acid

ferment, called variously uricase, uricolase and uricolytic ferment, which can obviously exert its activity only in the presence of oxygen. The existence of the ferment in particular glands of the lower animals has not been questioned, but the identity of the destruction product has been a subject of frequent discussion, different experimenters having from time to time assumed this to be each of the four substances—urea, glycocoll, allantoin and oxalic acid. Any one of these four compounds might have been expected since they are all well-known chemical decomposition products of uric acid:—



But the most recent investigations, undertaken with a knowledge of all possible sources of error and executed with improved methods, conclusively prove that allantoin is the only one of these four substances which is formed in the physiological destruction of uric acid.



As the present status of the case, regarding both the localization of the ferment and the products of its activity, is at variance with the earlier conclusions, it is necessary to assume that the latter are involved in error and explain them away on the most probable grounds.

The disappearance of uric acid of course does not necessarily mean its physiological destruction. The substance is very insoluble and easily lost in heat coagula. Moreover, the addition of alkali for the purpose of avoiding such loss may itself cause a destruction of the substance (Austin [1907]), and it is probable that in spite of a degree of protection which the proteins afford (Mitchell [1907]) this laboratory destruction of uric acid has actually been the ground for assuming the presence of uricase in tissues, which do not really contain it. Such cases have been pointed out by Wiechowski [1909] and by Folin and Denis [1913].

Accurate methods for the detection and determination of glycocoll in tissue extracts have not been available, while urea is a constant constituent of animal tissues. Indirect and almost valueless methods

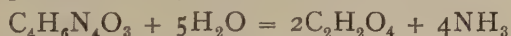
have therefore been employed for the detection of the one substance, while the formation of urea has of necessity been assumed only from quantitative differences observed. One may get an idea of the errors into which indirect determinations of glycocholl may lead by consulting the contribution of Hirshstein [1907] and its criticism by Samuely [1907] and by Abderhalden and Guggenheim [1909].

But perhaps the most convincing evidence against the formation of urea or glycocholl in the physiological destruction of uric acid is the fact that the very tissues in which such a formation was originally assumed, have been found to produce allantoin from uric acid and more nearly quantitatively as the analytical methods have been improved. At present the production of allantoin from uric acid is so well assured that the formation of any other substance would necessitate the assumption of two methods by which uric acid is physiologically destroyed.

Oxalic acid has so seldom been obtained by the physiological destruction of uric acid that one is inclined to look upon the substance as a product of laboratory decomposition. Claus [1874] found that by the action of potassium permanganate in the cold uric acid is quantitatively converted into allantoin:—



but if the products are even warm during the reaction, the allantoin is further decomposed, forming oxalic acid:—



This not only intimates the secondary origin of oxalic acid but furnishes a reason why experimenters have not generally found the theoretical amount of allantoin among the destruction products of uric acid.

The presence of uricase is therefore indicated either by the destruction of uric acid or the production of allantoin, but it is proven only when both phenomena simultaneously occur.

Allantoin was found in cow's urine by Wöhler in the year 1849.

In 1854 Frerichs and Städeler found allantoin in the urine of dogs with artificial dyspnoea induced by breathing small quantities of chlorine or by dropping oil into the lungs, but the substance could not be found in the urine of patients with dyspnoea or pneumonia. They did not know that allantoin is a normal constituent of dog's urine, and as they supposed its appearance to be connected with an insufficient supply of air they could not have regarded the substance as a decomposition product of uric acid.

Six years later Stockvis [1860] observed that uric acid is destroyed

when digested with gland extracts of the lower animals and found urea among the destruction products. The results of Stockvis were not generally known for forty-five years or until they were confirmed by Brunton and Bokenham (Brunton [1905]).

Meissner [1868] found allantoine in the normal urine of dogs, cats and rabbits, and Salkowski [1876] isolated allantoine from dog's urine after giving uric acid. The substance was identified by analysis, and it is certain that Salkowski regarded the allantoine as a destruction product of the administered uric acid.

Ascoli [1898] found that surviving dog's liver destroys perfused lithium urate and observed that the perfusate contained urea. The context of his publication proves the work to have been hurriedly done and the urea was not identified; it was only *quantitatively estimated*. Besides, one naturally expects to find urea in a fluid with which a surviving liver has been perfused. Ascoli's work is severely criticized by Wiener [1899].

At this time appeared the contemporaneous and independent contributions of Cohn [1898] and Minkowski [1898]. Cohn found a small amount of allantoine in the urine of dogs on a meat diet, but after feeding thymus allantoine was produced in so great a quantity as to be deposited from the urine when it cooled. After feeding two pounds of thymus, 1.9 gm. of pure allantoine were obtained from the urine, and, after feeding eighteen pounds of thymus, in the course of three days Cohn isolated from the collected urine, 18.4 gm. of a mixture of allantoine and uric acid from which 5.23 gm. of pure allantoine were prepared. But Cohn was unable to find allantoine in *human* urine after feeding thymus.

Minkowski's experiments gave results just as conclusive as those of Cohn. He fed dogs with pure nucleic acid prepared from salmon sperm and found a marked increase in the output of both phosphoric acid and uric acid:—

	P ₂ O ₅ in twenty-four hours' Urine.	Uric Acid in twenty-four hours' Urine.
Before nucleic acid	0.124 gm.	0.153 gm.
After nucleic acid	0.4379 "	0.945 "

But in spite of this marked increase, the amount of uric acid did not nearly correspond to the amount of nucleic acid employed. The same was the case after feeding thymus; a marked increase in the output of uric acid but not enough to satisfy theoretical requirements.

The reason for this discrepancy appeared when Minkowski obtained a crystalline deposit of allantoin after acidifying the urine with acetic acid. Having thus found that the uric acid of dog's urine is partly replaced by allantoin, Minkowski fed dogs with free hypoxanthine and found 77 per cent. of the substance in the urine as allantoin and 4 per cent. as uric acid. In consideration of the poor analytical methods at Minkowski's disposal these results suggest that allantoin is the only substance formed from uric acid in the dog's organism. But similar experiments with man led to no such results. After feeding hypoxanthine the uric acid was so far increased as to be deposited from the urine spontaneously, but allantoin could not be found. This is very striking. Allantoin is frequently found as a normal constituent of dog's urine but does not appear in human urine even under the most favourable conditions for its production.

Following Minkowski, Wiener [1899] found that the extracts of certain organs of the lower animals contain a thermolabile ferment, under whose influence uric acid is destroyed at the body temperature. He prepared thin aqueous pastes of the ground organs and submitted them to digestion at 40° in closed flasks with a preservative, and during the digestion the vessels were kept mechanically agitated to secure contact of the organ paste with the confined air. Determinations of uric acid were made with fractional parts of the material both before and after digestion, and after digestion with various purine derivatives. It was found that certain organs produce uric acid, showing that the formation of uric acid was in no way connected with putrefaction as Horbaczewski [1889] had supposed, but is due entirely to ferments present in the fresh tissues, whose activity is exerted in the presence of a preserving agent. The formation of uric acid was specially marked in the case of ox liver and ox spleen :—

<i>Ox Liver Paste.</i>	
Uric acid before digestion	0.0 mg.
Uric acid after digestion	59.4 "
Uric acid after addition of 200 mg. of hypoxanthine	116.3 "
<i>Ox Spleen Paste.</i>	
Uric acid before digestion	7.5 mg.
Uric acid after digestion	27.5 "

Unfortunately Wiener obtained equally good results with the thymus :—

<i>Calf's Thymus Paste.</i>					
Uric acid before digestion	1.7 mg.
Uric acid after digestion	22.8 "

But calf's thymus does not contain xanthine-oxidase. Spitzer [1899] found an extract of the gland incapable of forming uric acid from xanthine, and Jones [1904, 1] found no uric acid among the products of the self-digestion of thymus. As Schittenhelm [1904-5] confirmed these results it is likely that Wiener was dealing with xanthine which is plentifully formed in the auto-digestion of this gland.

But while ox liver and ox spleen produce uric acid certain other organs were found to destroy uric acid under the same conditions. Wiener observed this to some extent with pig's liver, dog's liver and ox muscle, but the destroying ferment exerted its greatest activity in ox kidney and horse kidney :—

Organ Paste.	Mg. of Uric Acid.	
	Before Digestion.	After Digestion.
Ox kidney	14.5	1.5
Ox kidney	5.8	0.0
Ox kidney after addition of 140 mg. of uric acid .	148.5	3.4
Horse kidney after addition of 130 mg. of uric acid	130.0	13.0

As the work of Stockvis [1860] was not generally known at the time, Wiener [1902]: [1903] believed that he had shown the destruction of uric acid by organ extracts for the first time. His work on the actual destruction of uric acid is most satisfactory but he did not find its destruction product. This he supposed to be glycocholl on rather insufficient grounds [1897], his principal reason for the assumption being that the decomposition products of uric acid protected the organism against the poisonous effects of benzoic acid, although some indirect chemical determinations of glycocholl were made.

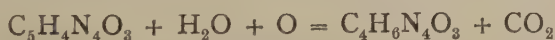
Following Minkowski metabolism experiments were made by Mendel and Brown [1900], who found allantoin in the urine of the cat after feeding pancreas, thymus or uric acid. Poduscha's [1900] wildly quoted failure to find allantoin in dog's urine after feeding uric acid was promptly explained by Swain [1901]; Luzatto's [1903] finding of oxalic acid in dog's urine after ingestion of allantoin was deprived of any importance by the work of Mendel and White [1904]; and little interest came to be felt in glycocholl, oxalic acid or urea, the attention of experimenters, being directed almost exclusively to allan-

toine as the only physiological destruction product of uric acid. Underhill and Kleiner [1908] found the substance present in the urine of starving dogs, and thus proved for the first time that allantoin has partly an endogenous origin, while Wiechowski and Wiener [1907] made a careful study of the conditions under which uricase exerts its activity in organ extracts (Wiechowski [1907]).

Wiechowski [1908] elaborated an excellent method for the quantitative determination of allantoin, in which the substance is weighed as such and which serves for the isolation of minute quantities.¹ By the application of this method he found allantoin so constantly present in the normal urine of the dog, cat, rabbit and monkey that he regarded the substance as the normal end product of purine metabolism in these animals. His results show that the amount of allantoin excreted by an individual on a purine-free diet is very constant in spite of wide variations in the output of total nitrogen, which is analogous with a similar observation of Burian and Schur [1900] on the excretion of uric acid by man.

An interesting communication of Hirokawa [1910] shows that nucleic acid fed to dogs produces an excretion of allantoin that is close to quantitative while the amount of uric acid is very small. But when thymus feeding has been continued for several weeks the allantoin gradually decreases and the uric acid increases until it becomes ten times its original amount.

Batelli and Stern [1909] adopted a unique method of estimating uricase which depends upon the formation of carbon dioxide in the destruction of uric acid:—



Errors in estimating allantoin are thus avoided. The tissues were found to stand in the following order:—

1. Ox kidney, most uricase.
2. Horse liver.
3. Cat's liver.
4. Dog's liver.
5. Rabbit's liver.
6. Horse kidney.
7. Various other organs of the lower animals, little uricase.
8. Human organs, no uricase.

Finally, Ackroyd [1911, 1]: [1911, 3] made perfusion experiments with surviving organs and found uric acid quantitatively converted

¹ For the modern method of estimating allantoin in urine, see Plimmer and Skelton [1914, 1]: [1914, 2]. Harding and Young [1919].

into allantoin by rabbit's liver. This result removes all doubt that was caused by Salkowski's [1902] experiments with rabbits.

The Purine Ferments of Human Organs.

When it was known that nuclein fermentation is brought about by the action of five independent physiological agents (nuclease, guanase, adenase, xanthine-oxidase and uricase), and that their localization is different in different animal species, it became desirable to learn their distribution in the organs of man.

The first investigations for this purpose were reported by Schittenhelm and Schmid [1907, 1]. At that time Schittenhelm was of the opinion that guanase and adenase are simply two terms for the same ferment which he called deaminase. Having found therefore that a given tissue could deaminize one of the amino-purines he saw no reason for making experiments with the other. Under these conditions Schittenhelm and Schmid reported the following experimental results :—

Human organ extracts which destroy uric acid.	Human organ extracts which deaminize guanine, adenine, or both.	
Kidney.	Kidney.	Spl��n.
Liver. ~	Liver.	Thymus.
Muscle.	Muscle.	Lung.
	Intestine.	

In their summary they state that they experimentally proved the existence of "deaminase" in seven human organs, and that the ferment undoubtedly is present in them all; that xanthine-oxidase exists nearly everywhere that cell nuclei are to be found; that uricase was experimentally proven in three human organs, but is seen to be present in all the organs from the disappearance of material in the experiments with guanine and adenine.

Xanthine-oxidase was subsequently stated to exist in human liver by K  nzel and Schittenhelm [1908].

There is no doubt that Schittenhelm and Schmid believed they had proven a uniform distribution of all the purine ferments in all human organs, but the experimental evidence upon which their conclusions were based is almost amusing. Various organ extracts were prepared, the purine base to be examined was introduced, and a current of air was passed through the mixture as it digested at 40  . At the end of the digestion the product was found to contain little or no purine material, from which it was concluded that deaminization and oxidation had led up to the formation and subsequent destruction of uric acid, and the presence of the necessary intervening ferments was assumed.

This assumed existence of uricase in human organs, without which the results of Schittenhelm and Schmid would be meaningless, found great service in the subsequent metabolism studies of Brugsch and Schittenhelm [1907]: [1909], who, after examinations conducted upon gouty patients, concluded that gout is an anomaly of the nuclein metabolism connected with a disturbance in the destruction of uric acid. "Es handelt sich also bei der Gicht um eine Anomalie des ganzen fermentativen Systems der Harnsäurebildung und Harnsäurestörung."

Following the publication of Schittenhelm and Schmid, Wiechowski [1909] reported that he was not able to demonstrate a destruction of uric acid by human organ extracts although special precautions had been taken against a post-mortem injury of his material.

Wiechowski's results were verified by Miller and Jones [1909] who had failed to find uricase in a gouty liver. As the other purine ferments of the gouty organs were found to have a markedly different distribution from the one which had been stated by Schittenhelm and Schmid, Miller and Jones supposed they were in possession of data to show that gout is indeed an anomaly of the purine fermentation, but subsequent examinations of normal cases failed to show the normal distribution different from that of gout.

There are two ways of dealing with a purine ferment. If its presence is to be proven in a tissue extract, the extract must be shown capable of decomposing a maximum amount of the added purine. But if the absence of a purine ferment is to be proven, the extract should be shown incapable of decomposing the minimum amount of the purine compound which is formed from the nucleic acid of the gland extract itself. Miller and Jones found uric acid among the *auto-digestion* products of human liver, although the digestion had been carried on under conditions favourable to its destruction. It is therefore safe to state that human liver does not contain uricase.

Examination of the organ extracts of four cases, typhus (Winternitz and Jones [1909]), gout, endocarditis, and aneurism (Miller and Jones [1909]), gave uniform results which show the following distribution of purine ferments in human organs :—

1. Uricase is not present in the human liver.
2. Adenase is not present in any human organ,¹ Adenine added

¹ An interesting article has appeared by Long [1913] who states that he can *occasionally* demonstrate the conversion of adenine into hypoxanthine by aqueous extracts of the mixed organs of human embryos at a certain stage of development. But much of his work with embryos and all of his work with adult organs is in agreement with the conclusion of Jones and his co-workers that human tissues do not contain adenase.

to digesting organ extracts could be recovered unchanged in as great an amount as should reasonably be expected. It is true that traces of hypoxanthine were formed, but this was also found when no adenine had been added and evidently results from the nucleic acid of the gland extract, not from the added adenine. This trace of hypoxanthine was called "preformed hypoxanthine".

3. Guanase is present in the kidney, liver and lung, but not in the spleen and pancreas.

4. Xanthine-oxidase is extremely active in the liver, but is not present in any other organ.

After the publications of Jones and his co-workers, Schittenhelm [1909] made a re-examination of human organs for the purine ferments with the following results:—

1. The general and often quantitative destruction of uric acid previously observed by Schittenhelm and Schmid could not be verified, uric acid being largely recovered after digestion at 40° with human organ extracts. Still there was some loss of uric acid which Schittenhelm considers too large to be ascribed to an analytical error, yet too small to be ascribed to ferment action.

2. Xanthine-oxidase is present only in the liver. The formation of uric acid can be conclusively shown only in this one organ, and here only from guanine (not adenine).

3. Adenine is converted into hypoxanthine by human tissue extracts incompletely and with difficulty. It can for the most part be recovered unchanged.

In this connexion a very interesting experiment is described with *muscle extract*.

350 c.c. of extract + 410 mg. adenine, digested at 35° for three days with passage of air.

Recovered 830 mg. of adenine picrate, equivalent to 308 mg. adenine. But in the filtrate from adenine picrate some typical crystals of hypoxanthine picrate were obtained which were carefully identified, "Es war also neben dem Adenin auch Hypoxanthin nachgewiesen" [1909, p. 266].

Surely it is not surprising to find hypoxanthine in a muscle extract. The recovery of 75 per cent. of the initial adenine is of considerably greater interest.

Schittenhelm gives the following explanation of the great differences between his results and those previously reported by Schittenhelm and Schmid: "Meine jetzigen Untersuchungen mit menschlichen Organen sind also wohl in Einklang zu bringen mit den früher in

gemeinschaft mit Schmid erhaltenen Resultaten." They can certainly be brought into agreement with the results reported in the meantime by Jones and his co-workers.

The Question of Uricase in the Human Organism.

The scattered literature on the subject suggests here and there that uricase is present in human organs, but a critical examination of the entire evidence leaves no room whatever to suppose that human beings have the power to destroy uric acid. It is curious that man should be the one animal to have lost so useful a function.

Poduschka [1900] "estimated" what he supposed to be allantoin in human urine, but he did not identify the substance.

Pfeiffer [1906] executed a similar procedure. The fact that he found the enormous amount of 1.9 gm. of allantoin per day in dog's urine shows wherein the trouble lies.

Croftan [1908] observed a destruction of uric acid by extracts of human organs but could find none of its assumed destruction products.

In Schittenhelm's later work [1909] the disappearance of small quantities of uric acid is easily accounted for by the action of the alkali which he employed, exerted over long periods, as Wiechowski has remarked [1909]. To say that uric acid does not suffer destruction in extracts of human organs because of the presence of "inhibitors" which *may* not exert their inhibition in the living organism, is scarcely to the point. Guanase and xanthine-oxidase act well enough in the organ extracts, and the best evidence against the presence of uricase in man has been acquired from studies of the living organism.

Burian and Schur's [1900] failure to recover more than half of the uric acid injected into men is without significance. Their analytical results are the lowest recorded in the literature. They certainly lost some uric acid: how much cannot be stated.

Frank and Schittenhelm [1909] concede that allantoin is the principal destruction product of uric acid in the lower animals, but claim that the human organism is exceptional in that it converts uric acid into urea. After feeding men with thymus nucleic acid they found a rise of about 10 per cent. in the excretion of urea, and drew the most remarkable conclusion that uric acid is no more an end product in the metabolism of man than of the lower animals.¹

¹ This is very clear in the light of the recent work of Thannhauser and Dorfmueller [1918, 2]. They show that in man the difference between the combined purine and the increased output of uric acid that should follow its administration is due to bacterial destruction of the purine ring in the alimentary canal. Hence the feeding of nucleic acid increases the urea of the urine,

The more obscure results of Landau [1909], Jacoby [1889], and Rotky [1910] add nothing to what has been stated.

Against these few negative results can be cited an array of evidence which practically amounts to proof.

v. Benezúr [1909] found parentally administered uric acid excreted unchanged.

Soetbeer and Ibrahim [1902] recovered uric acid quantitatively in the urine after injection into men.

Loewi [1900]: [1901] found the ratio of excreted uric acid to phosphoric acid in man constant after feeding with thymus nucleic acid.

The recovery of uric acid after digestion with extracts of human organs has been so conclusively established as to leave no room for debate (Wiechowski [1909], Miller and Jones [1909], Wells and Corper [1909]).

Batelli and Stern [1909] have observed that the organ extracts of the lower animals which form allantoin from uric acid, simultaneously produce carbon dioxide:—



But extracts of human organs do not produce carbon dioxide under the same conditions; independent evidence is thus furnished that human organ extracts contain no uricase.

Hunter [1916] was able to isolate allantoin from the blood of lower animals but could not obtain the substance from human blood.

Wiechowski [1910] applied to human urine a method for the determination of allantoin so accurate as to serve for the estimation of a few milligrams of the substance. By this method he had demonstrated the quantitative conversion of uric acid into allantoin in the living organism of the lower animals, and in aqueous extracts of their organs [1907]: [1908], thus showing that the destruction of uric acid is not attended by the production of any other nitrogenous substance (urea, glycoll). Wiechowski supposed he would be able to prove the absence of allantoin from human urine and thus end all discussion of uricase in the living human organism. But he found 10-15 mg. of allantoin in a day's human urine. This was a little annoying to Wiechowski as he was certain of this trace of allantoin and still was not inclined to give it any great importance. Its amount was so small that human urine could easily be distinguished from that of the lower animals by its poverty in allantoin.¹

The absence of uricase in the human organism was finally proved

¹ By the great preponderance of evidence it is therefore to be concluded that the end product of nuclein metabolism in man is uric acid, but in the lower animals allantoin.

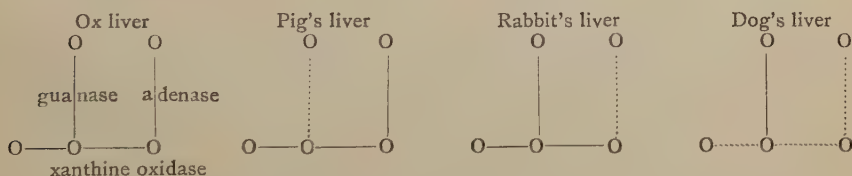
by Ackroyd [1911], who found appreciable quantities of allantoin in common foods. This is the origin of the 10 mg. of allantoin per day in human urine. The history of the subject shows how long an obvious error can be defended.

The Distribution of the Purine Ferments.

A great deal of space would be required to consider the distribution of these ferments for investigations have been very wide, extending even to invertebrates (Mendel, and Wells [1909]), but a few of the more interesting cases may be mentioned.

1. The purine ferments do not appear simultaneously but are formed successively as embryonic development proceeds (Jones and Austrian [1907, 2], Mendel and Mitchell [1907], Jones and de Angulo [1908], Wells and Corper [1909]).

2. The independent existence of guanase, adenase and xanthine oxidase as well as the variation of their distribution with species is strikingly shown by an examination of the livers of four animals. Jones and Austrian [1906] found that ox liver forms uric acid from both amino-purines, pig's liver from only one (adenine), rabbit's liver only from the other (guanine), and dog's liver from neither. The results are shown in the following diagrams which are abbreviations of that on page 74, the absence of a ferment being indicated by a dotted line:—



These results were looked upon by Jones and Austrian as an expression of the ability of the organ extracts to form uric acid *from nucleic acid*. But this is not necessarily true, for it has been found that certain gland extracts may decompose a free purine base and not decompose the base when it is in nuclein combination (Jones [1911, 2], Amberg and Jones [1911, 2]).

3. Uricase may be regarded as a liver ferment since it is probably present in the livers of all the lower animals except the ape (Wells and Caldwell [1914]) (ox, dog, pig, sheep, rabbit, guinea-pig, horse, rat, opossum, monkey), and except for an occasional occurrence in the spleen (ox) the ferment is found only in the liver. Its location makes it very effective so that allantoin is far more abundant than uric acid

in the urine of the lower animals. This appears in the analyses of the urine of seventeen animals, twelve of which were made by Hunter and his associates (Hunter [1914]: Hunter and Givens [1912]: [1914, 1]: [1914, 2], Hunter, Givens and Guion [1914]). They calculate a factor for each animal species called the "uricolytic index" which is directly proportional to the allantoin and inversely proportional to the uric acid. The following table, adapted from that of Hunter and Givens [1914, 2], shows the great preponderance of the allantoin over the uric acid in the urine of the lower animals, in contrast to the urine of man and the ape:—

Animal Species.	Uricolytic Index.
Opossum	79
Sheep	80
Horse	88
Monkey	89
Goat	92
Cow	93
Guinea-pig	94
Rabbit	95
Raccoon	95
Rat	96
Coyote	97
Cat	97
Dog	98
Badger	98
Pig	98
Ape	0
Man	0

4. Xanthine oxidase like uricase is generally confined to the liver (ox, pig, rabbit, guinea-pig, opossum, man) but is not so widely distributed as uricase. Thus certain livers (rat and dog) are provided with a ferment to destroy uric acid but with none to form it. This is not an uncommon circumstance. Rabbit's liver is able to oxidize hypoxanthine to uric acid but cannot form hypoxanthine from adenine.

Perhaps the most active occurrence of xanthine oxidase is in human liver which accords with man's low output of purine bases, the ratio of purine bases to uric acid being thirty-five times greater in monkey's urine than in human urine.

The deficiency of xanthine oxidase in the organism of the monkey (*cercopithecus*) was noted by Hunter [1914]. In a haphazard quantity of the urine he found—

Uric acid	*320
Xanthine	*950
Hypoxanthine	*360
Guanine	*000
Adenine	*000

Even subcutaneously injected xanthine was recovered unchanged (Hunter and Givens [1914, 1]).

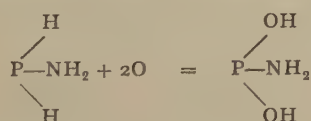
Xanthine oxidase is not present in yeast where such a multitude of ferments occur : nor is uric acid to be found in plants.

5. Guanase is the most widely distributed of all the purine ferments. With many animal species it is uniformly present in all of the principal organs (rat, ox, guinea-pig, rabbit). But pig's organs are peculiarly deficient in the ferment, and the muscles of the animal frequently contain deposits of guanine (Virchow [1866]), due perhaps to "guanine gout" (Virchow [1866, 2]). Pig's urine contains guanine (Pécile [1876]), and the purine bases are always in excess of the uric acid (Mendel and Lyman [1910]).

6. Adenase on the contrary is very rare having a distribution that is somewhat complimentary to that of guanase. Its presence cannot be shown in any of the principal organs of the rat, man or rabbit. As the two ferments are seldom or never associated with one another, it seems queer that they should ever have been thought identical.

Muscular hypoxanthine, which forms a considerable part of what Burian and Schur [1900] call "endogenous" uric acid, is not the result of the action of adenase on adenine. Leonard and Jones [1907] were not able to observe a transformation of adenine into hypoxanthine by aqueous extracts of muscle, while Voegtlin and Jones [1910] found that perfused adenine is not altered by surviving muscle.

But the path of adenine metabolism does not always pass through hypoxanthine. None of the organs of the rat exhibit adenase (Rohdé and Jones [1910]) and Nicolaier [1902] found that in rats subcutaneously injected adenine is oxidized but reaches the kidney without deamination where it forms concretions of 6-amino-2-8-dioxy-purine :—



Ebstein and Bendix [1904] found a similar transformation of adenine in the organism of the rabbit. But these two are the only authentic cases in the literature where oxidation of a free amino-purine was found to occur without deamination. The agreement between the results of Nicolaier and those of Rohdé and Jones furnishes evidence to show that in the living organism the transformation of the purine bases is no different from that produced by the sum of the forces in the individual organ extracts. The question has been discussed by Jones [1910] in some detail,

7. The distribution of the purine ferments is often obscure because a given tissue extract may be able to bring about the decomposition of a combined purine but unable to effect a similar decomposition of the free base. Thus dog's liver cannot convert free adenine into hypoxanthine but it can form hypoxanthine from nucleic acid with the greatest ease. Human tissues do not contain adenase, yet the subcutaneous injection of adenosine causes a marked increase of uric acid (Thannhauser and Bommers [1914]).

A purine base may even undergo both deaminization and oxidation while still combined. Benedict [1915, 1] has shown that about 90 per cent. of the uric acid of ox blood is in combined form. It is present only in the corpuscle and is set free by a ferment present, when the blood is allowed to stand. This contrasts sharply with the uric acid of chickens' blood which does not have a purine precursor. Here the uric acid is all free and in the plasma.

Bass [1914] found that the purine bases of human blood are combined and can only be detected after acid hydrolysis. He was able to isolate adenine but at most only traces of guanine.

8. The purine metabolism does not always suggest evolutionary relations, but it often does. The proof that uricase is not present in the tissue extracts of either the ape or man, and that allantoin is not present in the urine of either species (Wiechowski [1912]), surely justifies all the labour that has been expended upon the purine metabolism. Both species also fail to exhibit adenase, and exhibit guanase irregularly in the various organs (Wells and Caldwell [1914]).

The gradation from man to ape to monkey in relation to adenase is interesting. Hunter and Givens [1914, 1] found that injected adenine was largely excreted unchanged in the urine of the monkey, *Cercopithecus*, and Hunter [1914] was able to show adenase in slight activity in organ extracts of a second monkey, *Cebus apella*. With organ extracts of a third monkey, *Macacus rhesus*, Wells (1910) was able to obtain a striking demonstration of adenase.

The distributions of the purine ferments in the organs of the rabbit and guinea-pig are coincident throughout (Mitchell [1909]).

9. The purine metabolism of the rat is curious. Jones and Rohdé [1910] found that neither the individual organs nor the combined organs of the rat exhibit xanthine oxidase in spite of the fact that they could show the plentiful presence of uric acid in rat's urine. They also found that the combined organs of the rat could not change hypoxanthine. This apparent contradiction is not different from many similar cases and could be accounted for by assuming that in

rats, uric acid is formed along a path that does not involve xanthine oxidase. But Ackroyd [1914] found that the injection of *hypoxanthine* causes an increase in the allantoin of rat's urine. This was a most puzzling matter until the work of Benedict [1916] appeared.

10. Benedict found that the Dalmatian coach hound excretes both allantoin and uric acid, and that when the urine of the animal is acidified with hydrochloric acid a crystalline deposit of uric acid is formed. Careful analyses of the dog's urine were made for both allantoin and uric acid, over a long period of time, and then uric acid was injected subcutaneously. This caused the expected rise in the allantoin, but the injected uric acid also appeared, and quantitatively. From these results Benedict concludes that "uric acid and allantoin are interrelated in metabolism in other ways than have heretofore been assumed".

If uric acid can "induce" allantoin in the organism of the coach dog, it is conceivable that hypoxanthine can "induce" allantoin in the organism of the rat.

Nuclease.

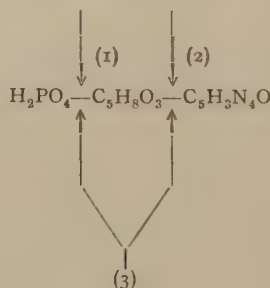
It was formerly supposed that nucleic acid is attacked by a ferment present in animal tissues so as to be completely decomposed into its final hydrolytic products. Two of these, phosphoric acid and purine bases, are easily detected, and it was commonly observed that they simultaneously appear when nucleic acids undergo enzymatic alteration. This idea was undoubtedly held by Pighini [1910], who was the first to apply the optical method to a study of nuclease. He observed a marked depression of the optical rotation of yeast nucleic acid when the substance is digested at 40° with the blood sera of rabbit, calf and man. As the optical rotation of yeast nucleic acid is markedly lowered when its solution is warmed to the body temperature (Amberg and Jones [1911, 1]), it is probable that the decline in rotation which Pighini observed was only in small part due to the action of the sera, and his results did not admit of interpretation so far as they concern the manner in which nucleic acid is decomposed, because he did not know the optical rotations of the possible decomposition products. Nevertheless, his observations were qualitatively confirmed by Levene and Medigreceanu [1911, 1] and by Amberg and Jones [1911, 1], who agree that the change brought about by the action of blood serum on yeast nucleic acid which alters its optical rotation, does not cause the production of either free phosphoric acid or free purine bases, and that blood serum does not exert any action at all on

thymus nucleic acid. Tscherroutsky [1912] has observed the same specific conduct of the two nucleic acids toward glucosidal enzymes. It is therefore highly probable that the decomposition of yeast nucleic acid by blood serum consists in the formation of mono-nucleotides.

The first systematic study of the several physiological factors which bring about the liberation of phosphoric acid and purine bases from yeast nucleic acid was reported by Levene and Medigreceanu [1911, 1]: [1911, 2]. They employed the optical method and experimented with various organ plasmata and with gastro-intestinal juices of the dog. By observing the optical changes of yeast nucleic acid when digested at 40° with these organic fluids, and knowing the optical constants of a number of the possible decomposition products, they could decide within certain limits the path along which the decomposition of the nucleic acid was proceeding. A simple case will illustrate.

Inosinic acid ($\alpha_D = -18.5^{\circ}$) may be decomposed in three ways:—

1. With formation of free phosphoric acid.
2. With formation of free hypoxanthine.
3. With formation of both substances.



In the first case inosine will be formed ($\alpha_D = -49.5^{\circ}$) and the rotation will rise.

In the second case d-ribose phosphoric acid will be formed ($\alpha_D = +4.4^{\circ}$). The negative rotation will fall to zero and then become slightly positive.

In the third case d-ribose will be formed ($\alpha_D = -18.5^{\circ}$) and the rotation will fall but remain negative.

It is thus possible to find the point of attack if only one reaction occurs and proceeds to completion.

With the complex nucleic acids of thymus and yeast the possibilities are great and inferences cannot be so sharply drawn, but after applying here and there supplementary chemical tests, Levene and Medigreceanu decided that the enzymatic decomposition of yeast nucleic acid occurs in three stages,

In the first phase, the tetra-nucleotide is decomposed into its four component mono-nucleotides by the action of a ferment called "nucleinase".¹

In the second phase the mono-nucleotides lose phosphoric acid and form the four corresponding nucleosides. As there is some reason to suppose that the pyrimidine nucleotides are decomposed by ferments different from those which cause the decomposition of the purine nucleotides, it may be necessary to assume the existence of four specific ferments. They are called "nucleotidases".

In the third phase the nucleosides are decomposed into their components, carbohydrate and base, and the ferment is called "nucleosidase".

The distribution of these three ferments in the body fluids and tissue extracts examined by Levene and Medigreceanu is as follows:—

Gastric juice and pancreatic juice contain none of these ferments, since they do not cause any change in the physical or chemical properties of nucleic acid.

Intestinal juice first forms the mono-nucleotides (nucleinase). The two pyrimidine nucleotides are not further changed, but the purine nucleotides are converted into nucleosides (nucleotidase).

Extract of intestinal mucosa forms the mono-nucleotides (nucleinase) all four of which lose their phosphoric acid and form the corresponding nucleosides (nucleotidase). The pyrimidine nucleosides are not further changed,² but the purine nucleosides are decomposed into d-ribose and purine bases (nucleosidase).³

Plasmata of kidney, heart muscle and liver exert an action similar to that of intestinal mucosa.

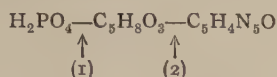
Pancreas plasma, blood serum and hæmolyzed blood proceed only as far as mono-nucleotides.

¹ Levene and Medigreceanu write familiarly of "nucleotides" and "nucleotidase," but at the time they wrote (1911) no nucleotide had been prepared from yeast nucleic acid, and no account could be taken of the influence that the optical rotations of the nucleotides would have on the results that they published.

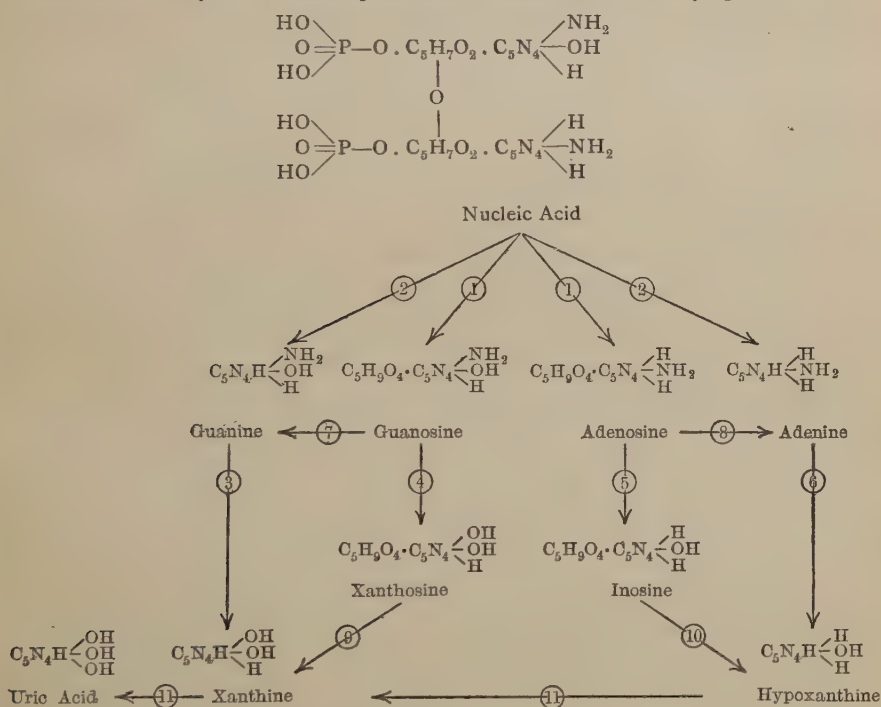
² The pyrimidine nucleosides are apparently not decomposed by any tissue extract (Levene and La Forge [1913]).

³ London, Schittenhelm and Wiener [1910-11]: [1911]: [1912] obtained results which led them to conclude that nucleic acid is neither digested nor absorbed by the stomach, but is decomposed in the intestine with intermediate formation of nucleotides and nucleosides. From the intestinal contents of a dog fed with thymus nucleic acid they isolated two substances which appeared to be guanylic acid and adenosine, and a third substance which they identified as guanosine. They thus found the decomposition products of *yeast nucleic acid*, but employed *thymus nucleic acid* in their experiments. Their results are chemically impossible.

different ways: phosphoric acid may be split off, forming the nucleoside, or purine bases may be set free. The two ferments involved are



called "phospho-nuclease" (1), and "purine-nuclease" (2). From this point the matter is very clear. The theoretical possibilities are coincident with the experimental results and are represented in the following diagram. The nucleic acid is represented as a di-purine-nucleotide. By the decompositions referred to it may give rise to



any or all of the four purine compounds, guanine, guanosine, adenosine and adenine.¹ This involves the hydrolytic action of—

(1) Phospho-nuclease.

(2) Purine-nuclease.

Any of the four amino-purine derivatives may undergo deaminization with production of the corresponding oxy-purine derivatives

¹ An active agent has very recently been found in the pancreas which is thermostable and can be tested after the other ferments of the tissue have been destroyed by heating [Jones, 1920, 2]. It decomposes yeast nucleic acid into its nucleotides. The active agent is not present in other organs than the pancreas, and does not decompose animal nucleic acid (see p. 46).

xanthine, xanthosine, inosine and hypoxanthine by the action of—

- (3) Guanase (Jones and Partridge [1904]).
- (4) Guanosine-deaminase (Jones [1911, 2]).
- (5) Adenosine-deaminase (Amberg and Jones [1911, 2]).
- (6) Adenase (Jones and Winternitz [1905]).

Any of the four nucleosides may undergo hydrolysis with the formation of the free bases by—

- (7) Guanosine-hydrolase (Jones and Belt).
- (8) Adenosine-hydrolase (Amberg and Jones [1913]).
- (9) Xanthosine-hydrolase (Jones [1911, 2]).
- (10) Inosine-hydrolase (Amberg and Jones [1911, 2], Levene and Medigreceanu [1911, 1]).

The joint action of these ferments leads up to the formation of the oxy-purines, xanthine and hypoxanthine, which are oxidized to uric acid by—

- (11) Xanthine-oxidase (Spitzer [1899], Wiener [1899]).

Uric acid is the end product of purine metabolism in man and the ape (Wiechowski [1912]), but is oxidized to allantoin in the organisms of the other lower animals by a ferment called uricase (Stockvis [1860], Wiener [1899]).

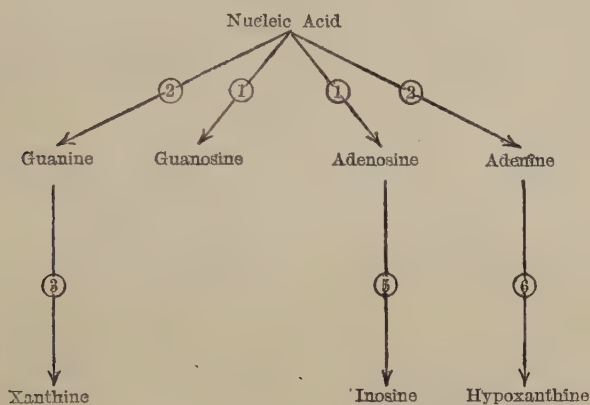
The general method of finding which of these ferments is present in an organ is as follows. Yeast nucleic acid is digested at 40° with an aqueous extract of the tissue in question and a determination of both phosphoric acid and free purine bases is made with part of the digested product. A second part of the digested product is then hydrolyzed with boiling sulphuric acid and the constituents again determined. From the data thus obtained it is in many cases possible to state what ferments the tissue extract contains. The particular results found with extract of pancreas will illustrate. By digestion with this tissue extract, yeast nucleic acid quantitatively loses its phosphoric acid and the digested product contains both free hypoxanthine and free xanthine. Upon hydrolysis of the digested product a relatively large amount of free guanine is produced and the free hypoxanthine is greatly increased.

The end products of the digestion were therefore—

- (1) Free xanthine.
- (2) Free hypoxanthine.
- (3) Combined guanine.
- (4) Combined hypoxanthine.

Remembering that combined purine bases under the conditions

must mean the corresponding nucleosides, it is clear that the end products of the digestion were xanthine, guanosine, inosine and hypoxanthine, and the ferments present in pancreas are therefore: guanase (3), adenase (6) and adenosine-deaminase (5), but not guanosine-deaminase (4), as shown in the following diagram (Jones [1911, 2]):—



The ferment distribution in dog's liver is of special interest. A number of animal tissues were examined by Jones and his co-workers which do not contain adenase. This was true of dog's liver, rabbit's liver, all organs of the rat and all human organs. But it was afterwards found that these tissues can form hypoxanthine from *combined* adenine. Amberg and Jones [1911, 2] showed that while dog's liver cannot form hypoxanthine from free adenine, it forms hypoxanthine quantitatively from yeast nucleic acid. The tissue evidently contains adenosine-deaminase (5), and inosine hydrolase (10), but neither adenosine-hydrolase (8) nor adenase (6).

Of the two possible paths leading from adenosine to hypoxanthine, one involves deaminization (5), followed by hydrolysis (10), and the other, hydrolysis (8), followed by deaminization (6). All animal tissues thus far examined can form hypoxanthine by one or the other of these two routes. The yeast alone is exceptional; it can deaminize neither free nor combined adenine (Amberg and Jones [1913]).

In the case of some tissues the experimental data are not sufficient for deciding the ferment distribution, but results have been obtained with one gland or another which justify us in postulating the independent existence of all the ferments that have been named.

The Purine Derivatives of Human Urine.

The origin of urinary uric acid has been the subject of numerous investigations which have furnished results upon which various conflicting theories of metabolism were founded. Horbaczewski's original suggestion that ingested substances increase the excretion of uric acid in proportion to the leucocytosis which they induce has been alternately supported and discredited by subsequent experimenters, while the results of metabolism experiments have been so divergent as to suggest that some important factor of uric acid excretion has been entirely overlooked.

In this connexion the results of Plimmer, Dick and Lieb [1909] are of considerable interest. They observed that ingested substances that give rise to an increased number of leucocytes cause an increase in the excretion of uric acid during the period that the leucocytes are present in the blood, not after they have disappeared. The explanation is offered that the increase of uric acid is not attributable to the nucleic acid of the dead leucocytes but is possibly a metabolic product of the living leucocytes. The idea is supported by some experimental evidence, but, as Plimmer, Dick and Lieb themselves observe, other paths by which uric acid may be formed are not excluded. Their results suggest a toxemia produced by the ingested substance, and one can easily imagine a disturbance at the same time of the forces whose action leads up to the formation of uric acid. In any event it has been conclusively demonstrated that ingested purine compounds are themselves transformed into uric acid but that the amount of excreted uric acid, except in very rare instances (Levinthal [1912]), does not nearly correspond to the purine content of the ingested substance. This discrepancy is in some cases accounted for as a loss of purine material in the fæces (Krüger and Schittenhelm [1902], Hall [1903]), but the wide variations in the results of different experimenters who by intravenous or subcutaneous injection have avoided any such error, precludes the supposition that the purine bases of the fæces can be a disturbing factor of considerable consequence.

It is not intended to convey the idea that all losses in metabolic studies have been caused by disturbances resulting from the presence in the organism of the ingested substance, but certain well-established phenomena are significant. The close approximation to quantitative results commonly obtained with nucleic acid and with hypoxanthine substances to which the organism is accustomed; the never-failing excretion of a considerable portion of ingested adenine by dogs, rabbits and men; the deposition of 6-amino-2-8-dioxy-purine after

injection of adenine into rats whose organisms are incapable of forming uric acid from adenine along the usual lines of adenine metabolism; the retention of guanine in the muscles of pigs whose organisms are poor in guanase; the marked alteration of metabolic conditions produced by the long-continued ingestion of purine material, are all matters that make desirable a study of purine metabolism as it is normally expressed in the composition of the urine. In the case of man such an examination leads to the establishment of a definite relation between the excreted purine derivatives and the localization of the purine ferments in the tissues. At the same time a new and interesting phase of purine metabolism is disclosed.

Fortunately an admirable analysis of human urine is available. From 10,000 litres of indiscriminately collected material Krüger and Salomon [1898]: [1898-99] prepared nearly 100 grm. of purine bases composed as follows:—

1-7-Dimethyl-xanthine (para-xanthine)	15'310 grm.
(Salomon [1883])	
1-Methyl-xanthine	31'285 "
7-Methyl-xanthine (hetero-xanthine)	22'345 "
(Salomon [1887], Krüger and Salomon [1895])	
7-Methylguanine (epi-guanine)	3'400 "
(Krüger and Salomon [1898])	
Xanthine.	10'110 "
Hypoxanthine.	8'500 "
Adenine	3'540 "
Guanine, absent	0'000 "
	<hr/>
	94'490 "

Krüger and Salomon noted that of 94'49 grm. of purine bases from human urine, 64'94 grm. are methyl-purines which are not directly obtainable from nucleic acid by chemical means. One might be at first inclined to believe that these methyl-purines are formed from the simple purines by methylation in the body, but a close examination of the matter strongly indicates that they are formed by de-methylation from substances containing a greater number of methyl groups.

The production in the body of mono-methyl-purines from di- and tri-methyl-purines was shown independently by Albanese [1895]: [1899] and by Bondzynski and Gottlieb [1895]: [1896]. The former found mono-methyl-xanthine in the urine of dogs after feeding caffeine or theobromine, and the latter obtained the same results with dogs, rabbits and men. The mono-methyl-xanthine in question was incorrectly supposed to be identical with hetero-xanthine, nevertheless the possession of de-methylating activity by the body was clearly proven.

Krüger and Schmid [1899] subsequently found that the supposed hetero-xanthine is in fact 3-methyl-xanthine, and proved that in dogs the methyl group in position three of the xanthine ring is most stable while that in position seven is most easily removed (Krüger and Schmid [1902]). In rabbits on the contrary the stability of the methyl groups is in reverse order to that in dogs, the methyl group in position seven being the most stable and that in position three the most easily removed :—

Substance Ingested.	Substance found in Urine in greatest quantity.	
IN DOGS.		
1-3-7-tri-methyl-xanthine (caffeine)	1-3-di-methyl-xanthine	Krüger [1899,1]
3-7-di-methyl-xanthine (theobromine)	3-methyl-xanthine	Krüger and Schmid [1899]
1-3-di-methyl-xanthine (theophylline)	3-methyl-xanthine	Krüger and Schmid [1902]
1-7-di-methyl-xanthine (para-xanthine)	1-methyl-xanthine	Krüger and Schmid [1899]
IN RABBITS.		
1-3-7-tri-methyl-xanthine	1-7-di-methyl xanthine	Krüger [1899, 2]
3-7-di-methyl-xanthine	7-methyl-xanthine	

There is good reason for supposing that in this respect the organism of man resembles that of the rabbit (Krüger and Schmid [1901]), and if this be the case the methyl-xanthines of the urine can easily be accounted for, since by removal of methyl group *three* from the methyl-purines of the food (tea, coffee, etc.) the methyl-purines of the urine are formed :—

Methyl-xanthines of the Food.	Methyl-xanthines of the Urine.
1-3-7-tri-methyl-xanthine (caffeine)	1-7-di-methyl-xanthine (para-xanthine)
1-3-di-methyl-xanthine (theophylline)	1-methyl-xanthine
3-7-di-methyl-xanthine (theobromine)	7-methyl-xanthine (hetero-xanthine) ¹

¹ Caffeine, discovery in coffee, Pelletier and Caventou [1825]; structure, Fischer [1881, 1]: [1881, 2]: [1882, 3].

Theophylline, discovery in tea, Kossel [1888, 2]; structure, Kossel [1889].

Theobromine, discovery in cocoa, Woskrensky [1842]; structure, Fischer [1882, 2]: [1882, 3]: [1897, 2].

Para-xanthine, discovery in urine, Thudichum * [1879], Salomon [1883]: [1885]; structure, Fischer [1897, 2].

Hetero-xanthine, discovery in urine, Salomon [1885]; structure, Krüger and Salomon [1895-96].

1-Methyl-xanthine, discovery in urine, Krüger and Salomon [1898]; structure, Krüger and Salomon [1898].

* See Salomon [1887, p. 415].

The presence of epi-guanine (7-methyl-guanine) in human urine may be explained on similar grounds as originating from some more highly methylated compound present in the food, especially as its methyl group is in the suspicious position seven. All that prevents a positive statement that the methyl-purines of human urine are derived from the methyl-purines of the food is the unaccountable presence of hetero-xanthine in *dog's* urine (Salomon [1887]).

The presence of the simple purines in human urine also admits of a very clear explanation. In the organism the purine groups of nucleic acid readily undergo deaminization with the final formation of the oxy-purines, xanthine and hypoxanthine, and this is the case whether the nucleic acid originates from the food, from metabolism of the cell-nucleus or from the bodies of dead leucocytes. To the oxy-purines thus formed is to be added the hypoxanthine formed in the living muscle. By the action of the xanthine-oxidase of the liver, the oxy-purines are oxidized to uric acid: but as the oxidizing ferment is present in only this one organ, an escape of small quantities of the oxy-purines is scarcely surprising, and their presence in the urine marks out the path along which uric acid has been formed.

But the food is partly autolyzed and contains traces of the *free* amino-purines to which must be added the free amino-purines formed from the food in the alimentary tract (Hall [1903]). Free guanine originating in this way will be partly found in the fæces and partly absorbed into the circulation where it will come in frequent contact with guanase and be completely changed to xanthine. But free adenine under these conditions should be excreted unchanged since adenase is not present in human organs.

Thus a consideration of the distribution of the purine ferments of human tissues would lead one to expect that the urine contains a relatively large amount of uric acid with much smaller quantities of xanthine, hypoxanthine and adenine, but no guanine. This is in strict agreement with the analysis of Krüger and Salomon.

The last word upon the origin of urinary uric acid is not likely to be spoken for some time, and many related questions will remain open to debate, but if one is inclined to believe that uric acid is not formed in the body from nucleic acid, he should at least note that the organism is equipped with a mechanism that can effect all of the transformations necessary to its formation.

APPENDIX.

Preparation of Thymus Nucleic Acid.

To a boiling mixture of 2 litres of water, 100 gm. of sodium acetate and 33 gm. of caustic soda is added in small successive portions 1 kg. of trimmed and finely ground thymus gland. The tissue usually dissolves completely forming a pale brown fluid, but any resistant portions are either removed or gotten into solution by heating for a short time over a small flame. The vessel containing the products is now immersed in a briskly boiling water-bath where it is allowed to remain with occasional stirring for two hours, when the product is diluted with one-third of its volume of water and made faintly but distinctly acid to litmus with 50 per cent. acetic acid. The amount of acid required is about 100 c.c., but the final additions must be made with care because the fluid will not filter unless the proper condition of acidity is reached. Any difficulty met at this point may be easily overcome by the alternate addition of acetic acid and sodium hydroxide and testing a small portion of the material after each addition on a small flat filter that has been heated with boiling water. When the acidity has finally been obtained which is favourable to rapid filtration, the material is heated to hard boiling and filtered with a hot water funnel. Under proper conditions, the filtration proceeds with considerable rapidity and continuously leaving a green slime on the filter and giving a pale yellow filtrate which gelatinizes upon cooling. The filtrate and washings are evaporated on a water-bath to about 750 c.c., and while warm the concentrated solution is poured slowly into 1000 c.c. of 95 per cent. alcohol. On standing overnight the precipitated sodium nucleate settles sharply to a spongy white mass from which the bulk of brown alcoholic fluid can be sharply decanted and the remainder pressed out with a spatula leaving the material in one cohesive mass. The substance is washed by decantation in turn with 80 per cent. and 95 per cent. alcohol, and after pressing out the last wash fluid as far as possible is transferred into a flask with 300 c.c. of hot water and heated on a water-bath. In half an hour or less, insoluble phosphates will collect leaving a perfectly transparent interstitial fluid which is treated with 10 c.c. of 20 per cent. caustic soda to lower the viscosity and filtered with a hot-water funnel. The perfectly transparent yellow filtrate is acidified with acetic acid and poured into 700 c.c. of 95 per cent. alcohol when sodium nucleate will be precipitated which can be washed by decantation as before with alcohol of increasing strength and ground in a mortar with absolute alcohol until it has crumbled to a fine white powder. If necessary, the absolute alcohol may be decanted and renewed once or twice

but not oftener, because the nucleate emulsifies with alcohol after the last traces of acetic acid and sodium acetate have been washed away. The material is finally washed to a filter with absolute alcohol and allowed to dry in a sulphuric acid desiccator. The yield of nucleic acid is about 33 gm. from 1 kg. of gland. The product is a fine white non-hygroscopic powder that can scarcely be improved by any method of purification. It is a soluble sodium salt of thymus nucleic acid but is generally referred to simply as thymus nucleic acid. Very similar or identical substances may be prepared by the same procedure from other animal tissues, rich in cell-nuclei (pancreas, spleen), but the method is not well adapted to the preparation of nucleic acid from yeast.

Nucleic acid is characterized by its decomposition products and by the viscosity and optical properties of its sodium salt. A 4 per cent. solution of sodium nucleate in warm water becomes gelatinous at the room temperature. With such a solution or one slightly more concentrated the following properties of the substance may be easily demonstrated:—

1. The viscosity of the solution is decreased by the addition of either acetic acid or sodium hydroxide. The solution may be changed back and forth from gelatinous to fluid by the alternate addition of acid and alkali.
2. The positive optical rotation is greatest in a neutral fluid and like the viscosity is markedly lowered by the addition of either acid or alkali.
3. The *specific rotation* falls by dilution with water, but more rapidly by dilution with acid or alkali.
4. The rotation is considerably lowered by heating a solution from 20°-40° but again rises when the solution cools.¹

Preparation of Yeast Nucleic Acid.

(a) *Baumann* (1918). "Fresh brewer's yeast is diluted with water and treated with a concentrated solution of sodium hydroxide (100 gm. of alkali per kilo. of yeast) and the solution then partially neutralized with hydrochloric acid. The solution is made acid and filtered from the separated protein. The clear filtrate is treated with 5 per cent. of magnesium sulphate and enough hydrochloric acid to cause the nucleic acid to flock out. The precipitated material is the monomagnesium salt and may be dried after washing with alcohol and ether. Yield: 4-7.5 gm. per kilo of yeast.

"The analyses for N, Mg and P are lower than the calculated figures, but the ratios of the three elements correspond accurately with those for monomagnesium nucleate."

(b) *Clarke and Schryver* (1917). "Thirty pounds of freshly pressed yeast are treated with a large excess of 95 per cent. alcohol, allowed to stand twenty-four hours, filtered on a Buchner funnel and dried in air. The air-

¹ These four properties of thymus nucleic acid were discovered by Jones and Austrian [1907, 1] and Jones [1908] and were stated as above in the first edition of this monograph 1914. Feulgen [1919] has recently rediscovered them.

dried material, in successive batches of 1000 gm. is boiled for two hours with 95 per cent. alcohol, filtered, pressed and dried in a current of air at 37°. It is then ground to a fine powder in a laboratory mill. One thousand gm. of finely ground material prepared in the above manner are extracted with 10 litres of 10 per cent. sodium chloride at a temperature 60°-80°. The extraction is continued with frequent stirring and intermittent heating to 60°-80° for four to five days. The residue is separated by pouring through muslin and pressing, the turbid fluid being filtered clear through paper. Ninety c.c. hydrochloric acid (1 pt. conc. HCl: 1 pt. water) are then added to the clear extract and the solution vigorously stirred. A characteristic precipitate of nucleic acid separates, quickly settling to a hard cake at the bottom of the vessel. After standing for two hours the liquid is siphoned off and the crude nucleic acid is washed with 50 per cent. alcohol till free from chlorine, left standing over night in 95 per cent. alcohol, and finally washed with absolute alcohol and ether. The yield is 15 gm. The crude nucleic acid thus obtained is a light brown powder and contains 7.8 per cent. of phosphorus. It gives no biuret reaction and has the characteristic properties of nucleic acid.

"The crude substance is best purified by a method described by Osborne and Harris (1902) by solution in warm 10 per cent. sodium acetate and precipitation with an excess of hydrochloric acid. Twenty gm. of the crude acid are dissolved in 500 c.c. 10 per cent. sodium acetate by warming for a short time on a water-bath. A small amount of insoluble matter is separated by centrifuging and a clear solution obtained, to which 100 c.c. alcohol and an excess of hydrochloric acid are added. The nucleic acid separates out as a white mass which settles quickly. It is well washed with 50 per cent. alcohol, 95 per cent. alcohol and ether, and finally dried in a vacuum. It is a perfectly white powder. There is practically no loss of material in this method of purification which gives a much whiter product than purification from dilute potash."

(c) *Levene and La Forge (1912)*. In stating the advantages of their method, Clarke and Schryver note that other methods involve the laborious preparation of nuclein which necessitates successive digestions of the material with pepsin and the consequent handling of large volumes of liquid. This objection, however, applies to the method of Levene and La Forge only in so far as this method is used for the preparation of triticonucleic acid from the wheat embryo: but when one is dealing with yeast there is no necessity for the previous preparation of a nuclein. The following abridgment of the procedure of Levene and La Forge may be employed with excellent results to the isolation of nucleic acid from yeast.

Caustic potash is ground, drop at a time, into a thick paste of compressed yeast and water until the reaction is faintly alkaline to litmus. A saturated aqueous solution of picric acid is then ground into the material in such amount as to thin out the paste and produce a deep yellow colour when the product is filtered on a Buchner funnel. Upon addition of hydrochloric acid

to the clear filtrate, nucleic acid is thrown out in its characteristic clot which soon adheres to the bottom and sides of the vessel. This product, slightly yellow from contamination with picric acid, is suspended in water, brought into solution with a very slight excess of caustic potash and after filtration from a small amount of insoluble material, the solution is acidified with acetic acid and poured into ten times its volume of alcohol. The precipitated nucleic acid when hardened and dried with absolute alcohol consists of a white powder which when dehydrated at 95° gives practically the theoretical values for both phosphorus and guanine.

However, the cost of compressed yeast is such as to preclude it as a source of nucleic acid and especially since a preparation is obtainable on the market so satisfactory as to leave little to be desired. For most work this commercial preparation will answer perfectly well, but if so desired it may be freed from its trace of protein by treating a concentrated solution of the substance in ammonia with a large excess of glacial acetic acid as described by Levene (1909).

The Analytical Chemistry of the Purine Derivatives.

The isolation of small quantities of the purine derivatives from complex mixtures such as the products of gland action, and their separation from one another would offer considerable difficulty if all five of the substances should be encountered at one time. But usually this is not the case. An amino-purine is either unaltered so that the corresponding oxy-purine does not come into consideration, or is completely deaminized when one is concerned only with the oxy-purine. Even under these simple conditions, however, the matter is not unattended with difficulty and requires some experience. In general an analytical scheme specially adapted to a particular problem is constructed from the following general propositions :—

1. All five of the purines are precipitated from dilute protein-free solutions by silver nitrate and ammonia in the cold (Neubauer [1867]) or by copper sulphate and sodium bisulphite at the boiling-point (Krüger [1894], Krüger and Wulff [1895], Krüger and Schmidt [1905]).
2. Guanine may be separated from the other three bases by its insolubility in ammonia (Schindler [1889]). It forms a useful analysable crystalline chloride $C_5H_5N_5O \cdot HCl \cdot 2H_2O$ that loses its water of crystallization sharply in a vacuum desiccator.
3. Adenine forms a very difficultly soluble picrate which serves for its separation from hypoxanthine (Bruhns [1890]), and a beautifully crystalline sulphate $(C_5H_5N_5)_2 \cdot H_2SO_4 \cdot 2H_2O$ that loses its water of crystallization very slowly below 120° .
4. Uric acid may be separated from any of the four bases by its insolubility in sulphuric acid (Horbaczewski [1894]).¹

¹ For the quantitative determination of uric acid, see Folin and Denis [1912]: [1913, 1]: [1913, 2]; Folin and Macallum [1912]; Folin and Wu [1919, 1]: [1919, 2]; Benedict [1915, 2]; Benedict and Hitchcock [1915]; Morris [1919].

5. Xanthine chloride is easily decomposed by water forming the insoluble free base, but hypoxanthine chloride is soluble in water without decomposition¹ (Krüger and Salomon [1898-99]).

In order to execute these methods successfully, one must be on his guard against certain sources of error (see Barnett and Jones [1911]).

The bases do not obey their individual laws of aqueous solubility in the presence of one another: insoluble xanthine for instance is easily soluble in an aqueous solution that contains guanine chloride (Krüger and Schittenhelm [1902]).

The method of Krüger and Salomon for the separation of xanthine from hypoxanthine though a most excellent one in experienced hands is otherwise quite useless.

Uric acid is easily lost both on account of its insolubility and its easy destruction by hot alkalis (Folin and Denis [1913]), two properties which have occasionally resulted in the supposed finding of a uric acid destroying ferment in animal tissues. But all these and other sources of error may be avoided without difficulty by consulting the recent literature upon the subject which is referred to in Part II. The matter is treated with some detail on p. 75.

A method for the quantitative estimation of purine bases in extracts of animal organs has been proposed by Burian and Hall [1903]. It is called the Method of Corrected Values and probably yields exact results, but on account of its complication has not come into very general use.

Preparation of Guanine and Adenine from Nucleic Acid.

25 grm. of commercial yeast nucleic acid are heated for one hour with 500 c.c. of 10 per cent. sulphuric acid in a flask provided with a simple condensing tube and immersed in boiling water. While still hot the pale yellow fluid is treated with concentrated ammonia, adding the reagent slowly after the precipitation of guanine begins, until the neutral point is roughly reached, when such an excess of ammonia is added that the fluid will contain about 2 per cent. of the reagent. Guanine is precipitated in heavy granular form while adenine remains dissolved. After filtering and washing with 1 per cent. ammonia (not water) the guanine is suspended in boiling water and brought into solution by the addition of the least amount of 20 per cent. sulphuric acid that will suffice. If too great an excess of sulphuric acid has not been used a small amount of animal charcoal will remove the colouring matter, leaving a perfectly colourless solution from which the base may again be precipitated at the boiling-point with an excess of ammonia. The precipitated guanine is filtered, allowed to dry at 40° and dissolved in 20-25 times its weight of boiling 5 per cent. hydrochloric acid. Upon cooling the solution deposits beautiful needle clusters of guanine chloride. The salt is the most useful compound of guanine. It crystallizes from extremely dilute solutions

¹ *Pure* hypoxanthine chloride is partly decomposed into the free base and hydrochloric acid.

in needles or slender prisms, having the composition $C_5H_5N_5O \cdot HCl \cdot 2H_2O$, and is unlike the chlorides of the other purine bases. It must be washed with very dilute hydrochloric acid (not with water) and dried in the air (not in a desiccator). From the pure chloride the pure base $C_5H_5N_5O$ may be prepared by dissolving in hot 1 per cent. hydrochloric acid and precipitating at the boiling-point with ammonia. Guanine prepared in this way by means of its chloride will be absolutely free from adenine, hypoxanthine and xanthine. The yield is about $2\frac{1}{2}$ gm. of pure guanine chloride from 25 gm. of nucleic acid.

By evaporation on a porcelain surface with a drop of dilute nitric acid a trace of guanine leaves a muddy yellow spot which changes to a brownish-red when moistened with caustic soda.

A very dilute solution of guanine chloride in $\frac{1}{4}$ per cent. hydrochloric acid does not give an immediate precipitate with picric acid, but upon standing for a while the solution suddenly becomes solid with beautiful deep yellow needles of guanine picrate.

Guanine is often met in association with magnesium ammonium phosphate. The substances are precipitated together by silver nitrate and ammonia and also by ammonia, so that specimens of guanine obtained from gland extracts are liable to contain phosphate. The phosphate, however, is not precipitated with the purine base in the procedure with copper sulphate and sodium bisulphite (see below) and is eliminated in the preparation of guanine chloride. A very satisfactory and common method of separating the two substances is to dissolve the base in warm dilute caustic soda, filter from the insoluble phosphate and precipitate the guanine from the alkaline filtrate by the addition of a slight excess of acetic acid.

The original ammoniacal filtrate containing adenine is joined with the various ammoniacal fluids obtained in the purification of guanine, filtered if necessary from a trace of guanine that may have been deposited on standing, and made faintly acid with 20 per cent. sulphuric acid. From this solution the adenine is precipitated at the boiling-point as adenine cuprous compound by the addition of 10 per cent. copper sulphate solution.

Precipitation of purine bases as cuprous compounds is the most important analytical operation connected with the substances and constitutes the first treatment to which a gland extract, urine or other impure mixture is submitted when it is desired to isolate uric acid or any of the four bases. When a boiling solution containing purine bases and an excess of copper sulphate is treated with a solution of sodium bisulphite (commercial saturated solution) there is first formed a white precipitate of copper-purine compound of the type $R \cdot Cu_2O$ which is practically insoluble in boiling water. The continued addition of bisulphite continually increases the white precipitate (so long as the copper sulphate is in excess) until all purine material has been precipitated when a further addition and boiling forms cuprous oxide and the white precipitate takes on a yellow cast. The addition of the reagent is then discontinued, the products are boiled hard for several minutes, filtered hot and

thoroughly washed with boiling water. Nucleosides (guanosine), colouring matters and various substances are removed and the purine bases are thus gotten into a condition of comparative purity.¹

The well-washed adenine-cuprous compound is suspended in hot water, decomposed with sulphuretted hydrogen, and the residue of adenine obtained by evaporation of the filtrate from copper sulphide to dryness on the water-bath is crystallized out of hot 5 per cent. sulphuric acid when adenine sulphate is obtained in crystalline form. If necessary the product may be decolorized by treating its solution in water with a very small amount of animal charcoal.

Adenine sulphate is easily soluble in hot water and when pure is very difficultly soluble in cold water so that little loss occurs in its purification after the first crystallization. The substance gives analytical results which accord sharply with the formula $(C_5H_5N_5)_2 \cdot H_2SO_4 \cdot 2H_2O$.

Soluble salts of adenine even in great dilution form a precipitate with picric acid consisting of needle clusters of the palest yellow colour. These may be purified by recrystallization from hot water. The picrate melts with decomposition at 270° . Adenine does not respond in the slightest degree to the xanthine colour reaction with nitric acid and caustic soda.

The Analytical Chemistry of the Pyrimidine Derivatives.

The separation of small quantities of the three pyrimidine derivatives from complex organic mixtures and from one another is not an easy matter. A method of isolating thymine from the hydrolytic products of proteins which was originally described by Jones [1900] has been found equally applicable to uracil (Ascoli [1900-1, 2]) and to cytosine (Kutcher [1903]). In this very commonly employed method the purine derivatives are first removed from an acid solution by precipitation with silver nitrate. Upon neutralization of the filtrate with barium hydroxide, the pyrimidines are precipitated as silver compounds insoluble in an excess of the reagent.

For the separation of the three pyrimidines from one another Levene [1903, 1] has made use of a scheme based upon the insolubility of thymine which admits of its isolation as such, and the difficult solubility of cytosine picrate by which the base may be separated from uracil.

This method does not effect a clean separation of thymine from uracil, nor is there any known method of making this separation. In his earlier work Kossel made frequent use of the fact that cytosine is precipitated by phosphotungstic acid while uracil and thymine are not, and Wheeler and Johnson [1907] have described a useful colour reaction for distinguishing uracil and cytosine from thymine but the analytical chemistry of the pyrimidine derivatives leaves much to be desired.

Identification of thymine and uracil can only be made by analysis

¹ The adenine solution under consideration contained d-ribose so that it was not necessary to use sodium bi-sulphite; but if a second precipitation of the adenine as cuprous compound is undertaken, sodium bi-sulphite must be used.

of the substances themselves, but cytosine forms an analysable picrate ($C_4H_5N_3O \cdot C_6H_3N_3O_7$), a chloroplatinate $C_4H_5N_3O \cdot PtCl_4 \cdot 2H_2O$ and a sulphate $(C_4H_5N_3O)_2H_2SO_4 \cdot 2H_2O$. The latter is prepared by the addition of alcohol to a concentrated solution of cytosine strongly acidified with sulphuric acid. The substance is slowly deposited in glistening crystals and there is no danger of forming the undefined basic sulphate if the cytosine solution is strongly acidified before adding the alcohol.

Preparation of Thymine and Cytosine from Thymus Nucleic Acid.

50 grm. of thymus nucleic acid are heated with 250 c.c. of 25 per cent. sulphuric acid in an autoclave for five hours at 150° - 160° . The product is diluted with water to a litre and hot saturated barium hydroxide is added to the boiling solution for the removal of phosphoric and sulphuric acids. More colouring matter is precipitated when the reagent is added in some excess and the excess removed with carbon dioxide. The yellow solution containing both purine and pyrimidine compounds is evaporated to about 400 c.c., acidified faintly with nitric acid and filtered from any purine compounds that are precipitated. The addition of silver nitrate will precipitate the remainder of the purine compounds together with most of the colouring matter, but thymine and cytosine will remain in solution. The continuation should be as rapid as is convenient and the solution kept cold with ice water. To the pale yellow filtrate from the purine silver precipitate is added successive portions of silver nitrate until a drop of cold saturated barium hydroxide produces a yellow precipitate signifying that enough silver nitrate is present to satisfy all of the organic compounds in the solution. The fluid is then treated with cold saturated barium hydroxide to faint but undoubted alkaline reaction and the precipitated pyrimidine silver compounds filtered off with a pump, suspended in hot water and decomposed with hydrochloric acid. To remove a trace of silver chloride which remains in solution the filtered fluid is treated for a short time with sulphuretted hydrogen and the filtrate from silver sulphide evaporated at 60° under diminished pressure to a small volume. Upon cooling or during the evaporation thymine is deposited in crystalline form and may be purified by recrystallization from hot water using animal charcoal. Its analysis corresponds to the formula $C_5H_6N_2O_2$ and the substance does not respond to the colour reaction for uracil.

The mother liquor from thymine is carefully evaporated to dryness to expel the greater part of the hydrochloric acid and the residue is taken up in cold water and filtered from a trace of thymine. The aqueous solution contains cytosine chloride which, after decolorizing with animal charcoal, may be used for the preparation of any desired derivative.

1. The addition of platinum chloride produces a crystalline precipitate of cytosine chloroplatinate $C_4H_5N_3O \cdot PtCl_4 \cdot 2HCl$.

2. Picric acid precipitates crystalline cytosine picrate $C_4H_5N_3O \cdot C_6H_3N_3O_7$.

3. Upon treatment of a moderately concentrated solution of cytosine chloride with ammonia the free base is precipitated in crystalline form and may be recrystallized from hot water. It forms plates with a pearly lustre of the composition $C_4H_5N_3O$.

Cytosine responds to the colour reaction of Wheeler and Johnson.

Preparation of Uracil and Cytosine from Yeast Nucleic Acid.

Yeast nucleic acid is heated in the autoclave with sulphuric acid and the product is treated exactly as described in the previous section to the point where the pyrimidine compounds are precipitated with silver nitrate and barium hydroxide. In this case the pyrimidine silver precipitate is suspended in hot water and decomposed with sulphuretted hydrogen. A small amount of barium present is quantitatively removed with sulphuric acid, and after concentrating the fluid a hot saturated solution of picric acid is added which slowly precipitates the cytosine, leaving the uracil in solution.

The cytosine picrate is recrystallized from hot water, dissolved in 5 per cent. sulphuric acid and the solution extracted in a separating funnel with ether for the removal of the picric acid. The resulting acid solution is concentrated on the water-bath and treated with an equal volume of alcohol. On cooling, beautiful crystals of neutral cytosine sulphate are deposited. These may be recrystallized as often as desired by treating a strong aqueous solution with sulphuric acid and alcohol.

The fluid containing uracil is freed from picric acid with sulphuric acid and ether, and after removal of most of the sulphuric acid with barium hydroxide, is evaporated to a small volume and allowed to stand. Impure crusts of uracil are deposited. Upon recrystallization, first from hot water using animal charcoal, and then from 5 per cent. sulphuric acid, the substance forms needle clusters, having the composition $C_4H_4N_2O_2$ which respond to the colour test of Wheeler and Johnson.

The Analytical Chemistry of the Nucleosides.

When yeast nucleic acid is heated in the autoclave at temperatures below 120° , the substance undergoes hydrolysis with the formation of nucleotides; but at higher temperatures the nucleotides lose their phosphoric acid and nucleosides are produced (Jones and German [1916]); (Levene and Jacobs [1916]). Therefore in preparing nucleosides the hydrolysis of nucleic acid is always done at the higher temperature (145° - 155°), in order that no residual nucleotide will complicate the isolation of nucleosides.

The principal analytical property of the nucleosides is their behaviour toward lead acetate. When a dilute neutral or faintly acid solution is treated with lead acetate, phosphoric acid and nucleotides are precipitated but the nucleosides remain in solution from which they may be precipitated as lead compounds by making alkaline with ammonia or barium hydroxide. From their lead compounds the nucleosides may be recovered.

Guanosine is very insoluble in water which makes the nucleoside easy of isolation and removal from solutions in which other nucleosides are to be sought. When purified by its lead compound as described, it forms crystalline needles having the formula $C_{10}H_{13}N_5O_5 \cdot 2H_2O$.

Adenosine forms an analysable picrate $C_{10}H_{13}N_5O_4 \cdot C_6H_2(OH)(NO_2)_3$ by means of which the nucleoside may be precipitated even from dilute solutions after the guanosine has been removed. This picrate has a very characteristic appearance, forming thin plates that glisten like spangles. Melting-point 183.5° (cor.). From the picrate, after purification by recrystallization, the free nucleoside may be recovered as a crystalline compound having the formula $C_{10}H_{13}N_5O_4 \cdot 1\frac{1}{2}H_2O$.

The two pyrimidine nucleosides are characterized by their resistance to hydrolytic agents so that before attempting to isolate them any purine nucleosides which are present may be decomposed with hot mineral acid and the purine bases removed as silver compounds. The pyrimidine nucleosides can then be isolated by means of their lead compounds.

Uridine may be prepared by such a procedure as crystals having the composition $C_9H_{12}N_2O_6$.

Cytidine forms a crystalline picrate and a difficultly soluble nitrate $C_9H_{13}N_3O_5 \cdot NO_3H$ which latter is the most useful compound of cytidine for analytical purposes. This nitrate may be purified to any desired extent by recrystallization and gives exact values for nitrogen when analysed by the modification of the Kjeldahl method for nitrates as described in the "Official Agricultural Methods" (with zinc dust and salicylic acid).

The nitrate seems to have displaced the picrate which was formerly used in the isolation of cytosine.

Preparation of Guanosine, Adenosine, Uridine, and Cytidine from Yeast Nucleic Acid.

Guanosine.—A solution of 100 grms. of commercial yeast nucleic acid in 500 c.c. of $2\frac{1}{2}$ per cent. ammonia is heated for $2\frac{1}{2}$ hours in the autoclave at 145° . After cooling, the product is allowed to stand over night in the ice chest when it becomes almost solid with gelatinous guanosine. This is filtered on a Buchner funnel, dissolved in hot water and treated with lead acetate for the removal of included phosphoric acid. After filtering off the lead phosphate, the filtrate is treated alternately with small portions of ammonia and lead acetate as long as either reagent produces an immediate precipitate in a filtered portion of the fluid. The precipitated lead compound of guanosine is filtered on a Buchner funnel, suspended in hot water and decomposed with sulphuretted hydrogen. The filtrate from lead sulphide is evaporated (preferably at 50° under diminished pressure) and the concentrated fluid allowed to stand. On cooling, guanosine is deposited in needle clusters which, upon recrystallization from hot water, form individual long thin plates with sharp edges. The crystals in minutest quantity respond to Tollens' pentose reaction with phloroglucine and hydrochloric acid.

250 mg. of guanosine are dissolved in 5 c.c. of 5 per cent. sulphuric acid and heated for half an hour just below the boiling-point. The cooled solution deposits crystals of guanine sulphate. These are filtered off and examined or redissolved by warming the solution. An excess of silver oxide is added, guanine-silver is filtered off, and the solution is made alkaline with caustic soda for the precipitation of a trace of silver. Upon addition of copper sulphate and boiling red cuprous oxide is precipitated.

Adenosine.—The filtrate from gelatinous guanosine described in the last section is faintly acidified with acetic acid if not already acid and treated with an excess of a 25 per cent. solution of lead acetate, and after filtration the nucleosides are precipitated as lead compounds by the alternate addition of ammonia and lead acetate. The lead precipitate is suspended in water, decomposed with sulphuretted hydrogen and the filtrate from lead sulphide is evaporated to a syrup, which is treated with a hot saturated aqueous solution of picric acid until cold saturated picric acid fails to give a precipitate with a filtered portion of the solution. The deposited adenosine picrate is recrystallized from hot water.

To prepare adenosine from the picrate, the latter is dissolved in hot water and the solution is made and kept acid to congo red as the picric acid is shaken out with ether. The sulphuric acid is then removed with lead carbonate and the filtered solution treated with sulphuretted hydrogen for the removal of a trace of lead. After evaporation of the filtrate from lead sulphide at 50° under diminished pressure and further concentration in a sulphuric acid desiccator, the fluid is allowed to stand in an ice-chest over night. A paste of crystalline adenosine deposits from which pure adenosine may be prepared in needle clusters by recrystallization from hot water with the use of animal charcoal.

When warmed with 5 per cent. sulphuric acid, adenosine rapidly undergoes hydrolysis with formation of pentose and adenine, as described above for guanosine.

Uridine.—A solution of 200 grm. of yeast nucleic acid in 1000 c.c. of 2½ per cent. ammonia is heated for two and a half hours at 145° in the autoclave, and after cooling in ice water the solution is filtered from guanosine with a pump as already stated. The filtrate is then evaporated in a porcelain dish on the water-bath to a syrup (75 c.c.) which is washed into a 2 litre distilling flask with 50 c.c. of boiling hot water and dissolved in 1½ litres of absolute alcohol.

The purine nucleosides are now destroyed by saturating the alcoholic solution with hydrochloric acid. As the gas is passed in, the temperature is allowed to rise nearly to the boiling-point of the liquid when the solution is cooled to the room temperature and the passage of hydrochloric acid continued to saturation. Before filtering, about one-third of the solution is distilled off and replaced by an equal volume of alcohol. This removes a sufficient amount of hydrochloric acid to produce a filterable liquid, so that the precipitated chlorides may be filtered off. This should be done with a Buchner funnel and

the precipitate must be thoroughly washed with alcohol. The combined filtrate and washings should measure about $3\frac{1}{2}$ litres.

The united alcoholic fluids are now treated with a hot alcoholic solution of lead acetate in small portions at a time until no longer acid to congo red, when 100 gm. of lead acetate in alcohol are added in excess. (In all, about 1 kg. of lead acetate dissolved in $1\frac{1}{2}$ litres of hot alcohol will be required.) The phosphoric acid and the greater part of the hydrochloric acid are thus precipitated. After filtration and thorough washing with alcohol, the fluid is evaporated in a vacuum to about 1 litre and then diluted with water to 3 litres which changes the alcoholic to an aqueous solution.

In order to remove the remainder of the purine bases, freshly precipitated suspended silver oxide is added until the presence of silver in the solution shows that an excess of the reagent has been used, and after standing over night the dirty silver precipitate is filtered off. Both silver and lead are removed from the filtrate with sulphuretted hydrogen, but before filtering, the excess of the latter is precipitated with a small amount of lead acetate. As the solution is now free from purine bases and phosphoric acid, the nucleosides may be precipitated as lead compounds. For this purpose 300 c.c. of 40 per cent. lead acetate are added and the nucleosides are precipitated by the careful addition of hot saturated barium hydroxide as long as the reagent produces a precipitate, *but an excess of barium hydroxide must not be used.*

The voluminous lead precipitate is filtered off with a pump, washed, made into a thick paste with warm water and decomposed with 25 per cent. sulphuric acid which is added drop at a time until a permanent acid reaction is shown with congo red.

The completeness of the reaction can easily be noticed in the changed appearance of the lead compound from light caseous lead nucleoside to heavy granular lead sulphate. Before filtering, the slight excess of free sulphuric acid is precipitated with lead carbonate which is added until the solution is no longer acid to congo red, and after filtering off the lead sulphate a trace of lead is removed from the solution by a short treatment with sulphuretted hydrogen. The solution now contains little besides the pyrimidine nucleosides. It is evaporated in a vacuum to a syrup which is taken up in 150 c.c. of warm alcohol. Upon concentrating this alcoholic solution to half its volume on the water-bath and cooling, it stiffens to a paste of crystalline uridine, a phenomenon that is greatly accelerated by scratching with a glass rod and if necessary treating with a little alcohol.

After cooling in the ice chest, the product is thinned out with alcohol and filtered. For purification, the crystals may be dissolved in 5 parts of 90 per cent. alcohol and the solution decolorized with animal charcoal. When the solution is evaporated to half its volume and cooled, uridine is deposited in large colourless crystals. From 200 gm. of yeast nucleic acid about 10 gm. of pure uridine are usually obtained.

The original alcoholic filtrate from uridine may be used for the preparation of cytidine (see below).

Cytidine.—To the cooled filtrate from uridine, 2 c.c. of cooled *pure* nitric acid and 25 c.c. of cooled alcohol are added. Cytidine nitrate ($C_9H_{13}N_3O_5 \cdot HNO_3$) is soon precipitated. By solution of the substance in a small amount of water and addition of alcohol acidified with nitric acid, beautiful colourless crystals of pure cytidine nitrate are thrown down. Melting-point 197° .

In this way, however, only a small amount of cytidine nitrate will be obtained (1 grm. from 200 grm. of yeast nucleic acid). For the preparation of the compound in quantity the following procedure may be used.

200 grm. of yeast nucleic acid are dissolved in 1000 c.c. of $2\frac{1}{2}$ per cent. ammonia hydrolyzed at 145° in the autoclave and both guanosine and adenosine removed as described. The filtrate from adenosine picrate is freed from picric acid by acidifying with sulphuric acid and shaking out with ether. The sulphuric acid is removed with barium carbonate and after evaporation to about 75 c.c. in a vacuum, the solution is treated with 10 c.c. of nitric acid and 75 c.c. of alcohol. In a short time the solution stiffens to a paste of cytidine nitrate which can be purified as described above.

The Analytical Chemistry of the Nucleotides.

When a solution containing a nucleotide is acidified with acetic acid and treated with lead acetate at the boiling-point, a lead compound of the nucleotide is precipitated in granular form which can easily be filtered: but as these lead compounds are quite soluble in boiling water the lead precipitate greatly increases as the solution cools.

This is the most important analytical property of the nucleotides and is generally employed as the first stage in their isolation and recovery. Nucleosides, and soluble constituents including various reagents, remain in the solution, but phosphoric acid, colouring matters, and various included impurities are carried down in the lead precipitate.

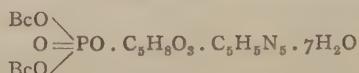
From the lead salts the free nucleotides may be prepared by suspending in warm water, decomposing with sulphuretted hydrogen and evaporating the resulting nucleotide solution to a syrup. By long contact with absolute alcohol and occasional grinding these syrups can be hardened to perfectly dry sandy powders, which are practically free from uncombined phosphoric acid and are little coloured since nearly all of the colouring matter originally present has been removed with the lead sulphide which is a most effective decolorizing agent.

There is a little danger that the crude nucleotide prepared in this way will contain sulphur so that the excess of sulphured hydrogen must be carefully removed before evaporating the filtrate from lead sulphide. This can be done by long aeration or by boiling the solution for a few minutes.

The crude nucleotides prepared in this way from their lead salts are all (except guanine nucleotide) syrups or amorphous powders contaminated with any impurities that are thrown down with alcohol. On the contrary, guanine nucleotide (when alone) is deposited from its hot concentrated aqueous solution

as it cools leaving various impurities in solution as in a crystallization. In fact guanine nucleotide differs at almost every point in its analytical properties from the other three nucleotides. Its ammonium salt is very difficultly soluble in 50 per cent. alcohol while the ammonium salts of uracil nucleotide and adenine nucleotide are quite easily soluble: a fact which is taken advantage of in the separation of guanine nucleotide from the other two.

By treating a hot aqueous solution of a crude nucleotide (1 : 3 or 4) with $2\frac{1}{2}$ parts of brucine dissolved in as little hot alcohol as will suffice for its solution, a brucine salt of the nucleotide is formed which is almost immediately deposited in crystalline form. All of the brucine salts are of the general type



The nucleotides are purified and separated from one another by fractional crystallization of these brucine salts from various solvents. For instance, the brucine salt of uracil nucleotide is very insoluble in cold 35 per cent. alcohol while that of adenine nucleotide is quite soluble.

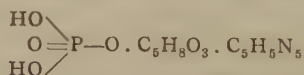
At first sight it would appear that the melting-points and analyses of these crystalline brucine salts would serve for the identification of the various nucleotides, but this is true only in very special cases. The molecular weight of uracil nucleotide is 224. The molecular weight of its brucine salt is 1138, so that the composition of the nucleotide is submerged in the composition of the brucine.

The same care has to be observed when dealing with the melting-points (except in the case of guanine nucleotide). The melting-points of various brucine salts of the following nucleotides show the limit of their usefulness:—

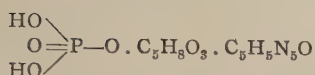
Adenine nucleotide	177 $\frac{1}{2}$ °
Adenine nucleotide and uracil nucleotide mixed	178°
Uracil nucleotide	178°
Uracil nucleotide and cytosine nucleotide mixed	178°
Guanine nucleotide	205°

It will be seen that the melting-points of the brucine salts is of use only in the case of guanine nucleotide.

When a brucine salt is suspended in hot water and the solution made alkaline with ammonia, the salt dissolves; but on cooling, brucine is deposited while the ammonium salt remains in solution. From the ammonium salt, the lead salt may again be prepared and then the free nucleotide. Upon evaporation in a vacuum of a solution of a nucleotide purified through its crystalline brucine salt the pure nucleotide is obtained. Adenine nucleotide is deposited in macroscopic needles which may be recrystallized from hot water. The analysis of these crystals is sharply in accordance with the formula



Guanine nucleotide is similarly deposited in granules as its concentrated aqueous solution cools.



But uracil nucleotide forms a syrup which may be hardened with absolute alcohol to a perfectly white powder.

Uracil and cytosine nucleotides may be separated from one another by a tedious fractional crystallization of a mixture of their brucine salts from 35 per cent. alcohol. From the brucine salt of each a crystalline barium salt may be prepared. When treated with nitrous acid in a Van Slyke nitrometer the nucleotides give off nitrogen very slowly and in accordance with the conduct exhibited by the nitrogenous group which the nucleotide contains. This will appear from the values in the following table which includes for comparison various purine and pyrimidine derivatives. The numbers are percentages of the amount of nitrogen required for one atom of amino-nitrogen (Van Slyke [1911], Wilson [1919]):—

Adenine	100
Adenosine	100
Adenine nucleotide	100
Guanine	108
Guanosine	127
Guanine nucleotide	120
Cytosine	135
Cytidine	100
Uracil	00
Uridine	00
Thymine ✓	00
Xanthine	a trace
Hypoxanthine	a trace

Preparation of Adenine Nucleotide from Yeast Nucleic Acid.

(A) By Hydrolysis with Ammonia.

A solution of 100 gramm. of commercial yeast nucleic acid in 530 c.c. of 2.5 per cent. ammonia is heated for one and a half hours in an autoclave at 115°, and the cooled product is treated with 530 c.c. of absolute alcohol. The bulky gelatinous precipitate thus formed is filtered on a Buchner funnel and drawn as completely as possible with a filter pump. The autoclave product is in this way separated into two fractions which may for convenience be designated as the guanine fraction and the adenine fraction. One fraction contains principally guanine nucleotide and forms an ammonium salt that is almost insoluble in 40 per cent. alcohol.

The aqueous alcoholic solution is acidified with acetic acid, diluted with an equal volume of hot water, and treated with an aqueous solution of lead acetate as long as the reagent forms a precipitate with a portion of the cooled and filtered solution. About 450 c.c. of 25 per cent. lead acetate are required. When the precipitation is complete the solution is cooled and the

heavy granular lead salt is filtered on a Buchner funnel and washed with a little cold water.

The lead salt is made into a thin paste with boiling water and decomposed with hydrogen sulphide. After the filtrate from lead sulphide has been carefully freed from every trace of hydrogen sulphide by boiling, it is evaporated at 50° under diminished pressure to about 50 c.c., and treated with a large amount of absolute alcohol. The precipitate is hardened with absolute alcohol and dried with sulphuric acid in a vacuum desiccator.

From 300 gm. of yeast nucleic acid 174 gm. of nucleotide are obtained.

Specimens prepared in the manner described always contain a small amount of guanine nucleotide and by acid hydrolysis produce a trace of guanine. The guanine nucleotide may be completely removed in the following way.

A solution of 100 gm. of the crude nucleotide in 200 c.c. of water is made alkaline with ammonia and treated with 300 c.c. of absolute alcohol. The filtered solution is diluted with an equal volume of hot water, acidified with acetic acid, and precipitated with lead acetate as described, but the lead compound is washed with water, until every trace of soluble ammonium salt is removed. This can be done easily and without using the immense volume of wash water that would otherwise be necessary, by grinding up the lead precipitate to a thin paste with boiling water and drawing off the cooled liquid as completely as possible with a filter pump. After the washing in this way has been repeated two or three times, the wash water fails to give off ammonia with sodium carbonate in the cold.

The lead salt is decomposed with hydrogen sulphide, the filtrate from lead sulphide, after boiling until free from the gas, is evaporated at 50° under diminished pressure and the pale yellow syrup is treated with a large excess of alcohol. The precipitated nucleotide is hardened and dried as described.

During the evaporation of the final aqueous solution a persistent bumping (due probably to the absence of ammonium salts) causes a serious loss of material so that the yield is lower than might be expected. From 100 gm. of the crude nucleotide 55 gm. of the purified product are usually obtained, or about 36 per cent. of the commercial yeast nucleic acid used. The theoretical yield from pure dry nucleic acid is about 50 per cent. The substance is an amorphous white powder, soluble in water in all proportions, but insoluble in absolute alcohol. It responds to the colour reactions for pentose with orcinic and phloroglucine, and by acid hydrolysis yields an abundance of adenine but no trace of guanine. It is a mixed nucleotide containing both adenine and uracil groups, but neither guanine nor cytosine groups.

A solution of 50 gm. of nucleotide in 200 c.c. of hot water is treated with a solution of 125 gm. of brucine in hot alcohol. Crystals of the brucine salt appear almost immediately. When the material has cooled to the room temperature, the crystals are filtered off, washed first with cold water, then with hot alcohol, and allowed to dry in the air.

50-60 gm. of this brucine salt are crystallized four times from 35 per cent.

alcohol and the four mother liquors united are allowed to evaporate slowly in a warm place. When nearly all of the alcohol has evaporated crystals of a brucine salt are deposited from the aqueous solution. This is principally the brucine salt of adenine nucleotide from which the free nucleotide may be prepared as follows:—

The brucine salt is suspended in 25 times its weight of boiling hot water (in which amount of water the substance does not completely dissolve) and made very faintly alkaline to litmus with ammonia. The suspended brucine salt goes completely into solution but no brucine separates. As the solution cools the careful addition of ammonia will throw out brucine in crystals that can be filtered easily.

The filtrate from brucine is extracted twice with small portions of chloroform, acidified faintly with acetic acid, concentrated under diminished pressure at 45° and treated with a slight excess of lead acetate. The lead compound is suspended in water, decomposed with hydrogen sulphide and evaporated to a small volume at 45° under diminished pressure. On standing over night the concentrated solution deposits a mass of crystalline needles which upon recrystallization from hot water form long transparent colourless rods.

Solutions of the nucleotide exhibit supersaturation to a remarkable degree. When a portion of the substance is dissolved in the smallest possible amount of boiling water, the solution may stand for several hours after cooling without depositing any crystals, but in a sufficient time (over night), practically all of the nucleotide crystallizes.

(B) By Oxidation with Potassium Permanganate.

A solution of 25 gm. of yeast nucleic acid in 250 c.c. of hot water with a few drops of caustic soda, is treated with 250 c.c. of hot aqueous solution containing 25 gm. of potassium permanganate. The solutions should not be boiling hot and the permanganate must be added in small portions at a time in order to avoid the occurrence of a violent reaction with evolution of gas. This phenomenon is very liable to happen but it will not occur if the conditions stated are observed.

After all the permanganate has been added the product is warmed until the manganese dioxide separates sharply leaving a perfectly clear pale yellow fluid. This is filtered with a pump and the black cake of manganese dioxide is extracted twice by grinding with 250 c.c. of boiling water each time. The united aqueous fluids without concentrating are heated to the boiling-point, faintly acidified with acetic acid and treated with a large excess of 25 per cent. aqueous lead acetate. After cooling, the lead precipitate is filtered on a Buchner funnel, thoroughly washed, suspended in warm water and decomposed with sulphuretted hydrogen. After removal of the excess of the reagent by aeration the solution is evaporated to a syrup at 45° under diminished pressure and hardened to a sandy preparation by long standing (3-4 hours) and grinding with alcohol. The substance is an impure mixed nucleotide

containing groups of both adenine and cytosine, but neither guanine nor uracil.

From this nucleotide the brucine compound is prepared by treating a hot aqueous solution (1 : 3 or 4) with a hot solution of $2\frac{1}{2}$ parts of brucine in a little boiling hot alcohol. The brucine salt is now purified by repeated recrystallization from hot 35 per cent. alcohol. After several crystallizations the substance forms beautiful transparent feathery needle clusters, which have the composition of "adenine-cytosine dinucleotide". (The Gunning-Kjeldahl method may be used for determination of any brucine compound, Jones [1919, 2].)

This purified brucine salt is now converted into the free nucleotides through the lead salts (as described in the section above). By concentrating the final aqueous nucleotide solution in a vacuum and cooling, needles of adenine nucleotide are deposited. (The substance is identical with the corresponding substance obtained by the ammoniacal hydrolysis of yeast nucleic acid.)

Adenine nucleotide is exceedingly insoluble in cold water so that the mother liquor is practically free from the substance. It contains a nucleotide that yields cytosine by strong acid hydrolysis.

Preparation of Guanine Nucleotide from Yeast Nucleic Acid.

(A) *By Hydrolysis with Ammonia.*

Portions of 100 gm. of yeast nucleic acid are heated with 530 c.c. of 2.5 per cent. ammonia in an autoclave at 115° for one and a half hours. After the product has cooled and while still alkaline with ammonia it is treated with 530 c.c. of absolute alcohol and allowed to stand until the supernatant fluid has become perfectly clear. The solution is now filtered and the grey precipitate, consisting principally of the ammonium salt of guanine nucleotide, is dissolved in hot water, filtered from a trace of insoluble flocculent material with a hot water funnel, and after the addition of a few drops of ammonia the cooled fluid is treated with two volumes of absolute alcohol. By alternate solution in hot water and precipitation with alcohol the ammonium salt of guanine nucleotide is thus freed from every trace of material that contains an adenine group. The effectiveness of the separation, however, must be ascertained by hydrolysis of a small portion of the substance with sulphuric acid and then testing for adenine. The ammonium salt is finally filtered with a pump and dried by grinding with absolute alcohol.

A solution of the ammonium salt in hot water is acidified with acetic acid and treated hot with neutral lead acetate as long as a precipitate is formed. After cooling, the lead salt is filtered off with a pump, suspended in hot water, and decomposed with sulphuretted hydrogen. The filtrate from lead sulphide is evaporated to a small volume at 45° under diminished pressure; upon cooling the concentrated solution in ice water, guanine

nucleotide is deposited. Upon repeated solution of the substance in hot water and deposition by cooling, every trace of soluble material can be removed, but the product still contains a small quantity of some insoluble substance which can be separated as follows. A concentrated solution of the mononucleotide in hot water is made alkaline with ammonia, boiled, filtered, acidified with acetic acid, and precipitated with lead acetate. The lead compound is decomposed with sulphuretted hydrogen, and the filtrate from lead sulphide is evaporated at 45° as described above. Without filtering, the cooled concentrated material is treated with absolute alcohol and the precipitated guanine nucleotide is washed and dried with absolute alcohol. The substance thus obtained is a snow-white amorphous powder, difficultly soluble in cold water, but easily soluble in warm water, forming a solution from which part of the guanine nucleotide is deposited on cooling in ice water. The substance is *laevo*-rotatory to polarized light.

(B) *By Ferment Action.*

A mixture of 200 grm. of commercial yeast powder (Lebedew [1911]), 2000 c.c. of water, 60 grm. of commercial yeast nucleic acid and 30 c.c. of chloroform is digested at 40° in a loosely stoppered vessel for three to five days or until a test of a few drops of the material with sulphuric acid shows that the nucleic acid has disappeared. While the product is still warm the clear yellow liquid is siphoned from the sediment and cooled in the ice chest when a pale yellow gelatinous precipitate will be deposited. In order that this precipitate shall be formed, the solution must contain a small amount of alcohol, so that if the yeast powder happens to be devoid of its alcoholic ferment (as is often the case with the commercial powder) it is necessary to add a small amount of alcohol to the products of digestion. The precipitate dissolves easily in hot water and is again deposited when the solution is cooled so that the substance can be obtained without difficulty as a perfectly white amorphous powder whose chemical composition accords closely with that of the guanosine salt of guanine nucleotide. The salt is dissolved in hot water and the hot solution is treated with lead acetate as long as a precipitate is formed. Guanine nucleotide is precipitated as a lead salt while guanosine remains in solution.

After thoroughly cooling or even standing over night, the lead salt is filtered off, suspended in hot water and decomposed with sulphuretted hydrogen. Upon evaporation of the filtrate from lead sulphide to a small volume at 60° under diminished pressure, guanine nucleotide is deposited as an amorphous powder. It dissolves easily in warm water and the solution has no tendency to gelatinize when cooled but again deposits guanine nucleotide in more or less granular form. It forms a beautifully crystalline brucine salt that melts at 205° and is in every way indistinguishable from the guanylic acid of animal glands.

To the filtrate from lead guanylate, lead acetate and ammonia are added

alternately as long as a precipitate of guanosine lead compound is produced. The lead compound is filtered, thoroughly washed, suspended in hot water and decomposed with sulphuretted hydrogen. Upon evaporation of the filtrate from lead sulphide at 60° under diminished pressure and cooling the concentrated fluid, guanosine is deposited in microcrystalline needle clusters. By recrystallization from hot water the substance forms beautiful individual crystals consisting of long thin plates with sharp edges. These macroscopic crystals have a composition expressed by the formula $C_{10}H_{13}N_5O_5 \cdot 2H_2O$ and respond to Tollen's phloroglucine test.

Demonstration of the Purine Ferments.

A ferment may be able to cause an alteration of a free purine base and yet be incapable of bringing about a similar change when the base is combined with carbohydrate as in the nucleic acid. Thus an aqueous extract of pig's pancreas converts guanine into xanthine rapidly and completely, but cannot convert guanosine into xanthosine. Two questions therefore arise in connexion with each purine base. The one is concerned with the ferment that acts upon the free base and is answered by an examination of the product formed when a relatively small amount of gland extract is digested with the free purine compound. In addition, the products are present which are formed by the action of the ferments on the nucleic acid of the gland extract, but the amount of extract is chosen so small that the purine bases coming from its nucleic acid can be neglected or they may be determined in a separate experiment. Usually, 400 c.c. of the gland extract are treated with 400 mg. of the purine base to be examined or an equivalent amount of one of its salts (600 mg. of either guanine chloride or adenine sulphate). In order to be sure that the purine base is in solution during the digestion and therefore accessible to the action of the ferments present it is well to dissolve it in the smallest possible quantity of warm normal caustic soda before adding it to the extract; but where a ferment is present in considerable activity this precaution is not necessary for the substance will undergo decomposition whether it is in solution or not. The prepared material is allowed to digest at 40° in a tightly closed vessel with enough chloroform to prevent putrefaction, but when tests are being made for xanthine-oxidase or uricase, air is frequently drawn through the digesting mixture from a wash bottle containing chloroform.

At the end of the digestion (12-48 hours) the product is diluted with water, heated to boiling and the precipitation of the proteins completed by the addition of a few drops of acetic acid. After filtering as rapidly as possible the boiling hot filtrate is treated as described below.

When hypoxanthine and adenine are involved no precaution is required at this point, but difficultly soluble guanine, xanthine and uric acid may be partly lost in the coagulum. If therefore one has reason to suspect the presence of any of these three substances it is well to make the heated digestion

products faintly alkaline and then acidify with acetic acid before filtering off the coagulated proteins : but this precaution is only required when one is dealing with a small amount of gland extract to which a comparatively large amount of purine compound has been added.

As stated, gland extracts are examined for ferments which act on combined purine bases. This is done by digesting the gland extract with nucleic acid or by examining the products formed in the self-digestion of the gland extract. In the one case 400 c.c. of the gland extract are digested with 2 or 3 grm. of sodium nucleate and in the other case, 2 or 3 l. of autodigested gland extract are employed. But in neither case is there much danger of losing any of the purine products in the coagulated proteins since uric acid, xanthine and guanine are more soluble in consequence of the greater amount of other organic compounds present. The filtrate from coagulated proteins is divided into two equal parts. In one part the free purine bases are determined by the method described below. The other part is treated with 100 c.c. of 20 per cent. sulphuric acid per litre and boiled for an hour, keeping at constant volume. At the end of the hydrolysis the sulphuric acid is nearly neutralized with caustic soda and the purine bases of the solution determined by the method described below. It can easily be shown that certain gland extracts liberate phosphoric acid quantitatively from nucleic acid (pig's pancreas). In these cases it is reasonable to assume that the additional purine bases produced by acid hydrolysis of the autolyzed product are to be referred to the corresponding nucleosides (guanine to guanosine, hypoxanthine to inosine, etc.).

A neutral or faintly acid, protein-free solution in which purine bases are to be determined is treated at the boiling-point with successive portions of 10 per cent. copper sulphate and commercial saturated sodium bisulphite as described on page 108, and the copper-purine precipitate is suspended in hot water and decomposed by adding a 1 per cent. solution of sodium sulphide. The copper sulphide which is produced does not subside, but the completeness of the reaction is easily noted by the production of a brown colour when a drop of the emulsion and a drop of lead acetate are allowed to run together on filter paper. The addition of a great excess of sodium sulphide must be avoided since its presence complicates the subsequent procedure. Acetic acid is now added to the emulsion as it boils until the copper sulphide collects, leaving a perfectly transparent interstitial fluid that can be quickly filtered. In moderately dilute solution, acetic acid does not immediately decompose the sodium salts of uric acid and xanthine, so that there is little danger of losing these substances in the copper sulphide if the work is done rapidly, but this is not true of guanine. Therefore when the presence of guanine is suspected sulphuric acid is used instead of acetic. One has now a faintly coloured solution of the purine compounds which is fairly free from other gland constituents and the separation of these substances from one another offers little difficulty : but it is advisable to construct an analytical procedure particularly applicable to the purine compounds supposed to

be present, which are indicated either by certain obvious limitations, by a test here and there of a drop of the material under examination, or, if necessary, by a preliminary experiment.

I. Uric acid is far in excess of the other compounds or is the only purine derivative present.—This condition occurs in examinations of urine and of digestion products of those glands which contain all of the uric acid forming ferments. The filtrate from copper sulphide is treated with 25 c.c. of concentrated hydrochloric acid and evaporated to 25 c.c. Uric acid separates from the solution during the evaporation in macroscopic colourless needles which respond beautifully to the murexide test. The mother liquor may be examined for traces of other purine derivatives by the methods that follow.

II. Uric acid and xanthine are the principal purine derivatives present.—The filtrate from copper sulphide is evaporated to dryness and the salts extracted with cold water. The residual mixture of uric acid and xanthine is dissolved in warm concentrated sulphuric acid (2 c.c. for every 100 mg.) and diluted with four volumes of water. The mixture is stirred until the uric acid begins to separate and then allowed to stand for three hours when uric acid will be almost quantitatively deposited while xanthine remains in solution. After filtration the fluid containing xanthine (or other purine bases) is strongly diluted, neutralized with caustic soda, and treated as before with copper sulphate and sodium bisulphite.

III. Xanthine is alone or with hypoxanthine, both guanine and adenine being absent.—The filtrate from copper sulphide is strongly acidified with hydrochloric acid and carefully evaporated to dryness on the water-bath with constant stirring at the end and the evaporation is repeated after moistening the residue. Under these conditions xanthine chloride loses hydrochloric acid and is present as the free insoluble base, but hypoxanthine chloride is not thus altered so that when the residue is digested with water at 40° the hypoxanthine chloride passes into solution leaving the xanthine. But the evaporation must be done with great care and over-heating of the dry products avoided. Otherwise part of the hypoxanthine chloride will decompose and hypoxanthine will remain with the xanthine.

a. The residue of xanthine is dissolved in 15 parts of warm 3·3 per cent. caustic soda and the solution is poured slowly, with constant stirring, into two-thirds of its volume of 32 per cent. nitric acid previously boiled until free from oxides of nitrogen. Upon cooling, xanthine nitrate is deposited in characteristic aggregates of small plates. By solution of the nitrate in dilute ammonia and evaporation on the water-bath crusts of pure xanthine are obtained.

When a trace of xanthine is evaporated on a porcelain surface with a drop of nitric acid a bright canary yellow spot is formed which has no suggestion of a red or pink shade (difference from uric acid). Upon moistening the yellow spot with caustic soda its colour changes to blood red without a suggestion of violet (difference from uric acid). Xanthine is quite easily soluble in 2 per cent. ammonia (unless it is crystalline) and very difficultly soluble in 5 per cent. sulphuric acid even on boiling (difference from guanine).

b. The solution of hypoxanthine chloride is diluted, treated at the boiling-point with copper sulphate and sodium bisulphite, and the copper hypoxanthine decomposed with sulphuretted hydrogen. After evaporation of the filtrate from copper sulphide to dryness the residue is crystallized out of 30 parts of colourless 6 per cent. nitric acid. Hypoxanthine nitrate is deposited in characteristic whetstone crystals which do not respond to the colour reaction for xanthine with nitric acid and caustic soda. Concentrated solutions of hypoxanthine salts form a crystalline precipitate of hypoxanthine picrate when treated with a solution of picric acid, but dilute solutions do not (separation of hypoxanthine from adenine).

IV. Guanine and adenine are present with or without xanthine and hypoxanthine.—The filtrate from copper sulphide (a drop of which may be tested for adenine with picric acid) is made alkaline with ammonia and the purine bases precipitated with a slight excess of an ammoniacal solution of silver nitrate. By suspending the well-washed silver precipitate in hot water and decomposing with hydrochloric acid a solution of the chlorides of the purine bases is obtained. The decomposition of the silver compounds goes very smoothly unless xanthine is present in the absence of guanine, in which case the silver chloride settles poorly leaving an emulsion of finely divided xanthine. This emulsion can be broken with an excess of hydrochloric acid but will not be formed if guanine is present.

In separating the four bases from a solution of their chlorides guanine and adenine must be removed before the oxy-purines. For this purpose the solution is heated to boiling and treated with an excess of ammonia. Guanine will be precipitated and can be converted into its chloride for purification (p. 107).

After boiling the filtrate nearly free from ammonia, it is faintly acidified to methyl-orange with hydrochloric acid and the adenine is precipitated with sodium picrate. Adenine picrate forms feathery needles of a very pale yellow colour which, after recrystallization from hot water, melt at 279° – 280° .

The picric acid is removed from the filtrate from adenine picrate with sulphuric acid and ether, the solution is neutralized with caustic soda and the oxy-purines are precipitated from the diluted solution at the boiling-point with copper sulphate and sodium bisulphite. As the amino-purines are now not present the precipitate of copper oxy-purines may be treated as directed in a previous section.

DEMONSTRATION OF ADENASE.

500 c.c. of aqueous extract of pig's spleen are digested at 40° with 400 mg. of adenine sulphate for a week. The adenine will have disappeared and hypoxanthine will be found in its place. Pig's spleen contains adenase.

500 c.c. of pig's spleen extract are similarly digested with 200 c.c. of guanine chloride. At the end of the digestion the guanine may be recovered unchanged while xanthine cannot be found. Pig's spleen does not contain guanase.

2 l. of pig's spleen extract (1 : 4) are digested at 40° for a week and the

product examined for purine bases. Guanine and hypoxanthine will be found. Pig's spleen can deaminate neither free nor combined guanine.

DEMONSTRATION OF GUANASE.

500 c.c. of an aqueous extract of dog's liver are digested at 40° with 200 mg. of adenine sulphate for three days. At the end of the digestion the adenine may be recovered unchanged and only a trace of hypoxanthine, which comes from the nucleic acid of the gland extract, will be found. Dog's liver does not contain adenase.

500 c.c. of dog's liver extract are similarly digested with 400 mg. of guanine chloride. The guanine will have disappeared and xanthine will be found in its place. Dog's liver contains guanase.

1 l. of dog's liver extract is digested at 40° with 5 gm. of yeast nucleic acid and the product examined for purine bases. Xanthine and hypoxanthine will be found. While dog's liver cannot deaminate free adenine, it can deaminate combined adenine. The tissue contains adenosine-deaminase and inosine-hydrolase.

DEMONSTRATION OF XANTHINE-OXIDASE.

500 c.c. of an aqueous extract of ox spleen is digested at 40° with 300 mg. of either xanthine or hypoxanthine for 24 hours. During the digestion the material is frequently vented with fresh air. At the end of the digestion uric acid, or a mixture of uric acid and xanthine, will be found. Ox spleen contains xanthine-oxidase.

DEMONSTRATION OF URICASE.

500 c.c. of an aqueous extract of ox kidney is digested at 40° with 400 mg. of sodium urate for 2 days and a current of air is passed continually through the digesting mixture. At the end of the digestion the product contains no purine derivative. The product is heated to boiling, filtered from coagulated protein and treated with an amount of lead acetate that will remove most of the phosphates but is not sufficient for complete precipitation. After filtering off the lead precipitate the fluid may be examined for allantoin by Wiechowski's (1913) method. Allantoin will be found. The ox kidney contains uricase.

DEMONSTRATION OF ADENOSINE-DEAMINASE AND THE ENZYMATIC PRODUCTION OF NUCLEOSIDES FROM NUCLEIC ACID.

2 l. of an aqueous extract of pig's pancreas are digested at 40° for a week with 15 gm. of yeast nucleic acid.

It can easily be shown that all phosphoric acid of the digested product is free and precipitable with magnesia mixture (Jones [1911, 2]). Therefore the nucleic acid must have been decomposed at least as far as nucleosides. When the digested product is cooled, gelatinous guanosine is deposited.

The material is divided into two equal portions, in one of which the

purine bases are directly determined. The other portion is boiled with sulphuric acid and the purine bases again determined. It will be found that xanthine and hypoxanthine are formed in the digestion and that subsequent hydrolysis produces additionally guanine and hypoxanthine. The end products of the digestion were therefore xanthine, hypoxanthine, guanosine and inosine, and the ferments of pig's pancreas are phospho-nuclease, purine-nuclease, guanase, adenase and adenosine-deaminase, but not guanosine-deaminase. The experiment can be done just as well with thymus nucleic acid.

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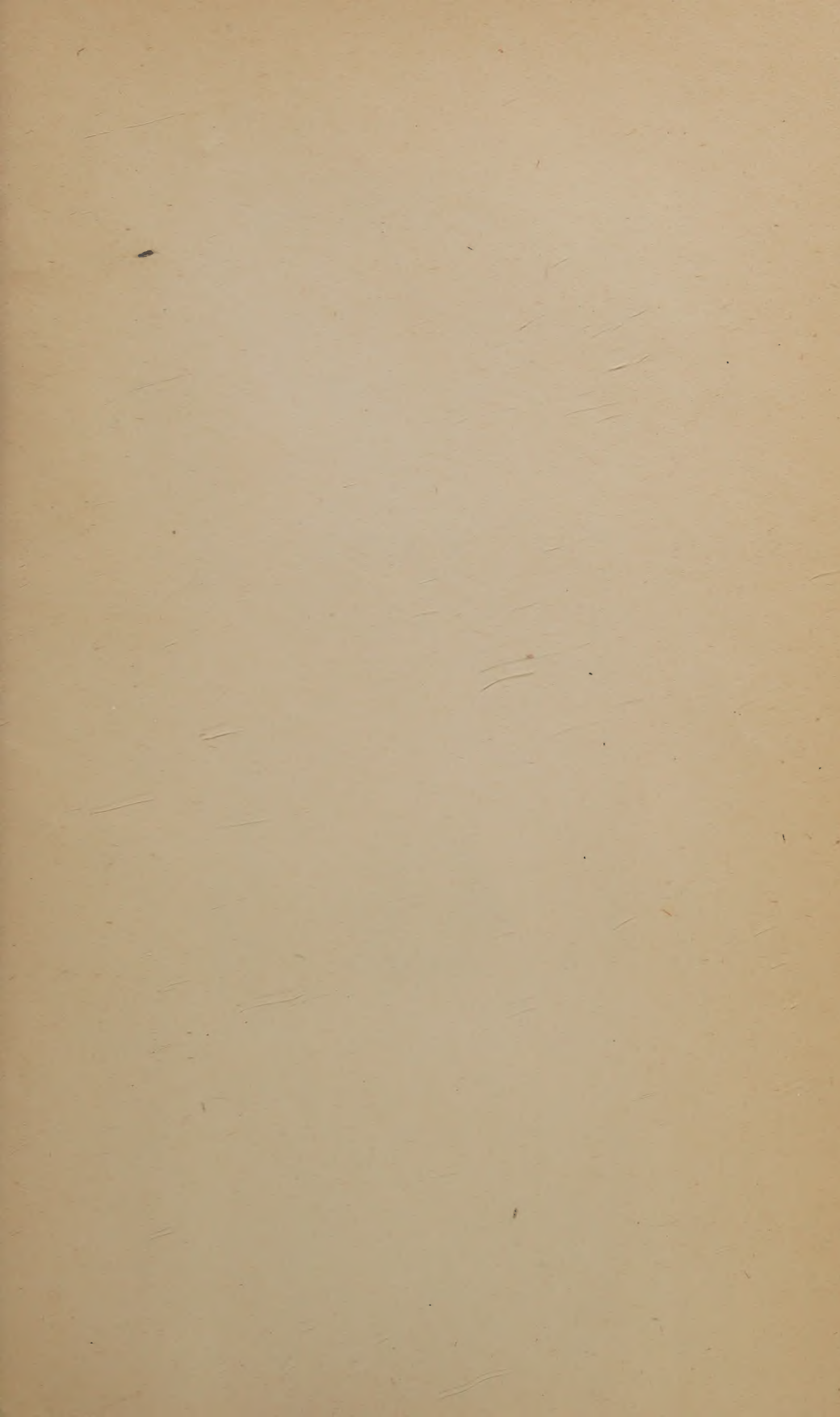
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